STUDIES ON THE EFFECTS OF METHOTREXATE AND FOLIC ACID ON THE GROWTH OF A TRYPANOSOMA,CRITHIDIA FASCICULATA(Dissertation_全文)

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CONTENTS

ABBREVIATIONS

Aminopterin,	4-amino-4-deoxypteroylglutamic acid
AMPte,	4-amino-4-deoxy-10-methylpteroic acid
Biopterin,	2-amino-4-hydroxy-6(1',2'-dihydroxypropyl)pteridine
Boc,	*t*-butyloxycarbonyl
Bz,	benzyloxy
CPase	carboxypeptidase
DFP,	diisopropylfluorophosphate
DFR,	dihydrofolate reductase
Dihydrofolate,	7,8-dihydropteroylglutamic acid
EDTA,	ethylenediamine tetraacetic acid
Folate,	pteroylglutamic acid
Homofolate,	homopteroylglutamic acid
Isoxanthopterin,	2-amino-4,7-dihydroxypteridine
Leucopterin,	2-amino-4,6,7-trihydroxypteridine
2-ME,	2-mercaptoethanol
MTT,	3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl tetrazolium bromide
MTX(methotrexate),	4-amino-4-deoxy-10-methylpteroylglutamic acid
NEM,	*N*-ethylmaleimide

Neopterin,	2-amino-4-hydroxy-6-(1',2',3'-trihydroxypropyl) pteridine
pABG,	*p*-aminobenzoylglutamic acid
pCMB	*p*-chloromercuribenzoic acid
PMSF,	phenylmethylsulfonylfluoride
POPOP,	1,4-*bis*-2-(5-phenyloxazolyl)benzene
PPO,	2,5-diphenyloxazole
Pteroate,	*N*-(2-amino-4-hydroxypteridine-6-ylmethyl)-*p*-aminobenzoic acid
PtR,	pteridine reductase
Pyrimethamine,	2,4-diamino-5(-chlorophenyl)-6-ethylpyrimidine
SDS,	sodium dodecyl sulfate
Trimethoprim,	2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine
Tris,	tris(hydroxymethyl)aminoethane
Xanthopterin,	2-amino-4,6-dihydroxypteridine
Z,	*N*-benzyloxycarbonyl

INTRODUCTION

As shown in Fig. 1, methotrexate (MTX) and aminopterin, 2,4-diaminofolate antagonists, are widely used for chemotherapies of cancers such as acute lymphocytic leukaemia, acute myelogenous leukaemia, choriocarcinoma, carcinoma of the head, neck, lung and breast, and of generalised psoriasis.[1-3] The therapeutic action is based on a potent or stoichiometric inhibition of dihydrofolate reductase (DFR, EC 1.5.1.3) in carcinoma cells.[1,4,5] DFR which catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate in the presence of NADPH has a key role in the synthesis of tetrahydrofolate and therefore in purine and thymidylate synthesis for providing DNA precursors.[1] Especially, thymidylate is synthesized *de novo* in the thymidylate synthetase (EC 2.1.1.45) reaction. Thymidylate synthetase catalyzes a novel reaction in which a methylene group is both transferred from 5,10-methylenetetrahydrofolate to C-5 of deoxyuridylate and reduced accommodatingly by oxi-

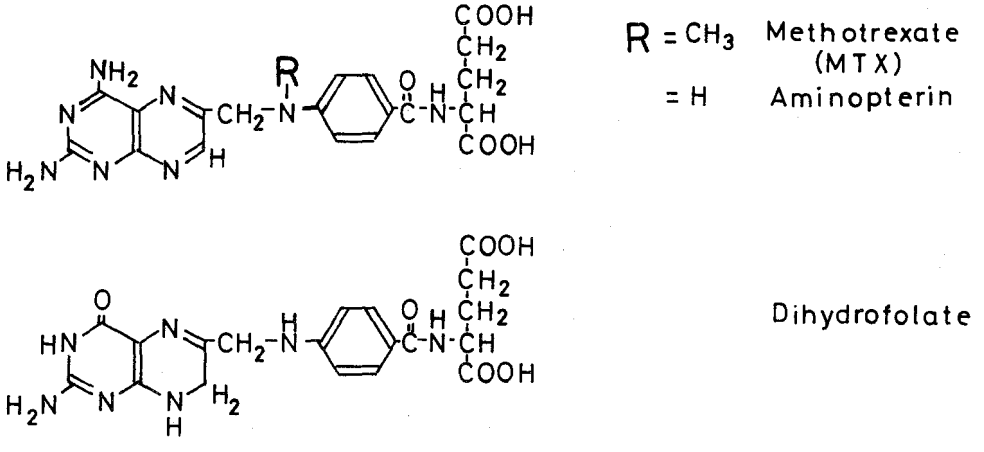


Fig. 1. Structures of MTX, Aminopterin and Dihydrofolate.

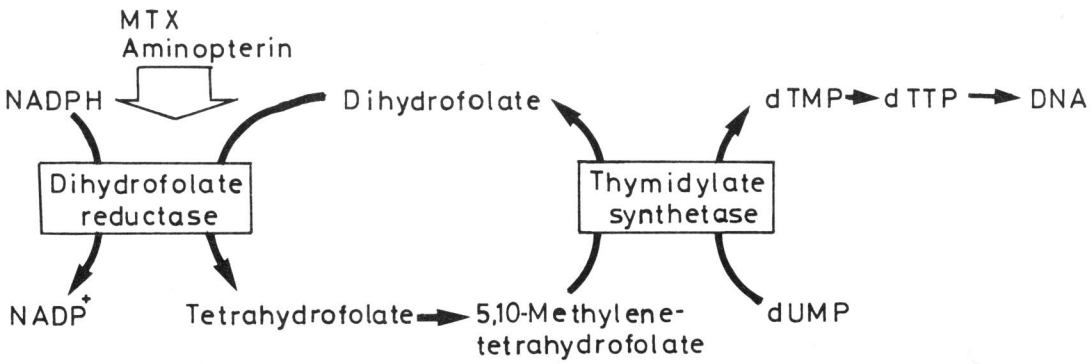


Fig. 2. Interrelation ship of Dihydrofolate Reductase and Thymidylate Synthetase

dation of the tetrahydrofolate to dihydrofolate[6-8] (Fig. 2). The dihydrofolate is reduced to tetrahydrofolate by the DFR reaction comprised the thymidylate-synthesis cycle[6,8,9] and reconverted to 5, 10-methylenetetrahydrofolate by the addition of the appropriate one-carbon unit. MTX and aminopterin as the effective inhibitors of the DFR reaction regulate the thymidylate-synthesis cycle and decline the DNA synthesis. Therefore, MTX and aminopterin are used not only for the chemotherapy of cancer, but also for elucidation of these reactions in various organisms.

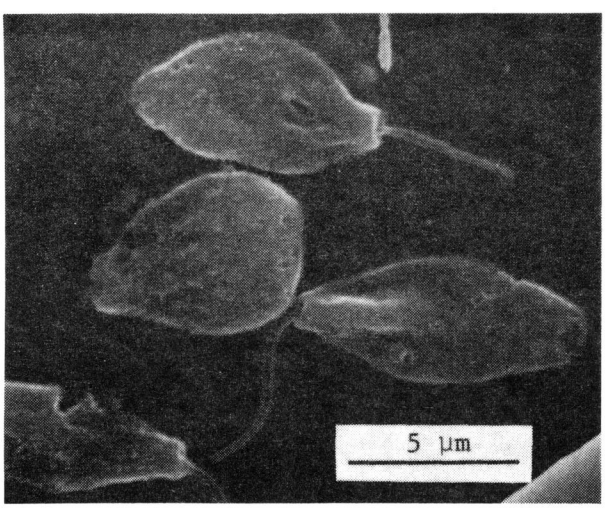


Fig. 3. Scanning Electron Micrograph of *Crithidia fasciculata*. A Hitachi Scanning Electron Microscopy S-450 was used.

The trypanosomid flagellate, *Crithidia fasciculata*, is a parasite of *Culex* and *Anopheles* mosquitoes, and its body is usually elongated and fusiform or pear-shaped with a length of 4 - 10 µm and a width of about 3 µm (Fig. 3).

This protozoan requires a small amount (0.4 - 21 nM) of a 6-alkylpterin such as L-*erythro*-biopterin and 6-hydroxymethylpterin.[10-12] It is noted for its usefulness as a biological system for assay of pteridine compounds (Table I).[13] This protozoan also requires unusually high amounts of folate (230 - 2,300 nM) for its growth.[10-12,14] However, the folate concentration required for growth is reduced to 1 - 11 nM, when a small amount of L-*erythro*-biopterin is present.[10-12] The author, preliminarily, found that MTX and aminopterin promoted the growth of *C. fasciculata* ATCC 12857 at the same concentrations as did folate, without showing any inhibitory effect.

Table I. Chemically Defined Medium of *Crithidia fasciculata*[13]

Compound	mg/L	Compound	mg/L
Casamino acids	10,000	Yeast Adenylic acid	50
L-Glutamic acid	3,000	Adenosine	1
L-Tryptophan	80	Guanine-HCl	1
DL-Alanine	200	Adenine-sulfate·$2H_2O$	1
DL-Methionine	100	Thymine	25
L-Tyrosine	100	Thymidine	1
L-Phenylalanine	100	Biotin	0.01
$MgCO_3$	1,000	Nicotinic acid	10
$CaCO_3$	20	Nicotinamide	0.5
K_3PO_4	50	Riboflavin	0.8
$FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$	70	Thiamine-HCl	2
$MnSO_4 \cdot H_2O$	31	Pyridoxine-HCl	1
$ZnSO_4 \cdot 7H_2O$	22	Pyridoxamine-2HCl	1
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	7.2	Pyridoxal-HCl	1
$CuSO_4 \cdot 5H_2O$	4	Ca-Pantothenate	4
H_3BO_3	0.57		
$CoSO_4 \cdot 7H_2O$	2.4	EDTA	300
$NaVO_3 \cdot 4H_2O$	0.46		
Sucrose	15,000	Hemin	25
K_3-Citrate·H_2O	2,000	Triethanolamine	5,000
Na_2-Succinate·$6H_2O$	1,000	Tween 80	3,000
Na-Acetate	365	Biopterin(Pteridine etc.)	

Adjust pH to 7.8 - 8.0.

In this thesis, in order to elucidate the metabolism of MTX and folate in *C. fasciculata*, the author first describes the promoting effect of MTX and aminopterin on the growth of this protozoan.

Secondly, the effect of MTX on the DFR activity of *C. fasciculata in vivo*, and purifications and properties of the two DFRs [DFR and pteridine reductase:dihydrofolate reductase (PtR:DFR)] activity in this protozoan are described. Thirdly, the uptake and metabolism of MTX by *C. fasciculata* and isolation of the metabolites of MTX are described. Fourthly, the author developes the radioassay for the activity of the new enzyme which catalyzes the hydrolysis of folate to pteroate and glutamate and is named tentatively as folate-hydrolyzing enzyme (FH-enzyme), and describes distribution of the enzyme in biological cells and tissues. Finally, the purification and properties of the FH-enzyme in *C. fasciculata* are described and a possible mechanism for metabolism of MTX and folate in *C. fasciculata* is also proposed.

REFERENCES

1) R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines," North-Holland Pub. Co., Amsterdam, London, 1969, p. 139 and p. 464.
2) J. R. Bertino, *Cancer Res.*, 23, 1286 (1963).
3) W. C. Werkheiser, *Cancer Res.*, 23, 1277 (1963).
4) A. L. Franklin, E. L. R. Stokstad, M. Belt and T. H. Jukes, *J. Biol. Chem.*, 169, 427 (1947).
5) W. C. Werkheiser, *J. Biol. Chem.*, 236, 888 (1961).
6) G. K. Humphreys and D. M. Greenberg, *Arch. Biochem. Biophys.*, 78, 275 (1958).

7) B. M. McDougall and R. L. Blakley, *Biochim. Biophys. Acta*, 39, 176 (1960).

8) E. J. Pastore and M. Friedkin, *J. Biol. Chem.*, 237, 3802 (1962).

9) R. B. Dunlap, N. G. L. Harding and F. M. Huennekens, *Annal . N. Y. Acad. Sci.*, 186, 153 (1971).

10) H. A. Nathan, S. H. Hutner and H. L. Levin, *Nature*, 178,741(1956).

11) G. W. Kidder and B. N. Dutta, *J. Gen. Microbiol.*, 18, 621 (1958).

12) H. A. Nathan and H. B. Funk, *Am. J. Clin. Nutr.*, 7, 375 (1959).

13) H. N. Guttman, "Pteridine Chemistry," ed by W. Pfleiderer and E. C. Taylor, Pergamon, London, 1964, p. 255.

14) H. A. Nathan and J. Cowperthwaite, *Proc. Soc. Exptle. Biol. Med.*, 85, 117 (1954).

Chapter I

Promoting Effect of Methotrexate and Aminopterin on the Growth of *Crithidia fasciculata*[a)]

It is generally known that MTX and aminopterin inhibit the growth of many bacterial and mammalian cells on the basis of a potent and irreversible inhibition of DFR.[1)] Preliminarily, the author found that the growth of *Crithidia fasciculata* ATCC 12857 was not inhibited, but rather promoted, by MTX and aminopterin at concentration of 0.1 mM. This chapter describes the promoting effect of MTX and aminopterin on the growth of *C. fasciculata*.

MATERIALS AND METHODS

Materials. Adenosine-3'-monophosphate (from yeast), aminopterin and hemin were obtained from Sigma Chemical Co. MTX was from Lederle Ltd. Vitamin-free acid hydrolyzed casein (Casamino acid) was from Nissui Pharmaceutical Co. Millipore filters (0.45 μm in diameter) were from Millipore Co. Guanine-HCl and adenine sulfate were from Kohjin Co., Ltd. Thymine, thymidine, adenosine, biotin, nicotinic acid, nicotinamide, riboflavin, thiamine-HCl, pyridoxal-HCl, pyridoxamine-2HCl, pyridoxine-HCl, calcium pantothenate, L-glutamic acid, L-tryptophan, DL-alanine, DL-methionine, L-tyrosine, L-phenylalanine and other chemicals were obtained from Nakarai Chemicals Ltd., Kyoto.

Medium and growth conditions for C. fasciculata. *C. fascic-*

ulata ATCC 12857 was grown at 25°C in a Coleman colorimetric cuvette (19 x 105 mm, round) containing the chemically defined medium (10 ml) described by Guttman[2] (see Introduction). MTX (0.1 - 100 μM), aminopterin (100 μM) or L-*erythro*-biopterin (0.04 - 1.7 nM) was aseptically added to the medium by filtrating through a Millipore filter. The cell growth was assayed turbidimetrically with a Coleman Model 6-20 Spectrophotometer and was shown by transmittance (T%) at 675 nm.

Preparation of cell-free extract of C. fasciculata. The cells grown in the chemically defined medium (100 ml) containing 1.5 nM L-*erythro*-biopterin or 0.1 μM MTX were harvested at the log phase (T=50%) by centrifugation at 3,000 x *g* for 5 min and washed thoroughly with 0.9% (w/v) saline. The cells were suspended in 5 ml of 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, and disrupted for 5 min on ice with a Kaijo-Denki 20 kHz Ultrasonic Oscillator. The supernatant solution obtained on centrifugation at 12,000 x *g* for 30 min was used for testing the DFR activity.

Assay method for DFR activity. The DFR activity in the cell-free extract of this protozoan was determined photometrically by the method described at the following chapter II.[3] The DFR activity was defined as the amount of tetrahydrofolate produced per min under the assay conditions.

Determination of protein. Protein was determined by the method described by Lowry *et al.*[4]

RESULTS AND DISCUSSION

Effect of MTX and aminopterin on the growth of C. fasciculata

The growth curve for *C. fasciculata* showed a lag as long as over 40 hr, even in the medium including 2 nM L-*erythro*-biopterin which was 20-fold higher than the concentration required for half-maximum growth.[5] As shown in Fig. 1, MTX (0.1 - 100 μM) and aminopterin (100 μM) promoted the growth-rate as well as L-*erythro*-biopterin (0.21, 0.85 and 1.7 nM). The growth curve obtained with the medium including 0.1 μM MTX coincided with that with the medium including 0.85 nM biopterin. As shown in Fig. 2, the addition of 100 μM MTX or aminopterin to the medium including 0.11 nM biopterin at 84 hr of cultivation caused the growth-rate promotion.

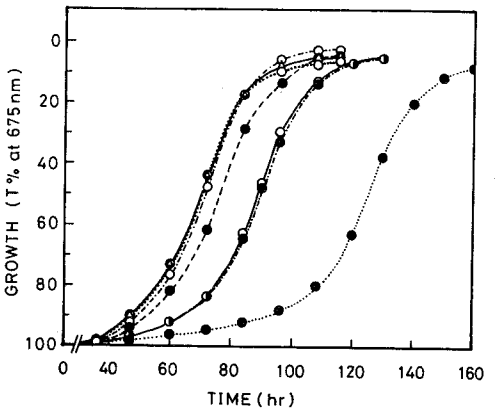


FIG. 1. Effect of Methotrexate, Aminopterin and L-*Erythro*-biopterin on the Growth of *C. fasciculata.*

C. fasciculata was grown at 25°C in the chemically defined medium containing one of the following compounds: methotrexate [0.1 μM (○——○); 1 μM (○-·-·-○); 10 ~ 100 μM (○------○)], aminopterin [100 μM (△——△)], or L-*erythro*-biopterin [0.21 nM (●------●); 0.85 nM (●-·-·-●); 1.7 nM (●---●)].

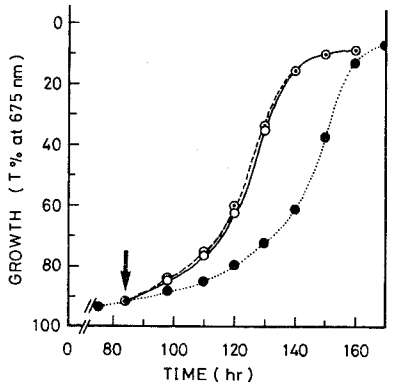


FIG. 2. Stimulating Effects of Methotrexate and Aminopterin on *C. fasciculata* Grown in the Biopterin-medium.

After 84-hr incubation (↓) in the medium containing 0.11 nM L-*erythro*-biopterin, 0.1 mM of methotrexate (○——○) or aminopterin (⊙---⊙) was added. (●------●), control growth with 0.11 nM biopterin.

The DFR activity in C. fasciculata Some mammalian and bacterial cells showing

TABLE I. DIHYDROFOLATE REDUCTASE ACTIVITY IN CELLS GROWN IN METHOTREXATE- AND BIOPTERIN-MEDIA

C. fasciculata was grown in the chemically defined medium (100 ml) containing 0.1 μM methotrexate or 1.5 nM L-*erythro*-biopterin at 25°C and harvested at the log phase (T = 50%). Dihydrofolate reductase activity was assayed by the photometric method described previously.[10] The reaction mixture contained 50 mM potassium phosphate, pH 7.0, 30 mM 2-mercaptoethanol, 80 μM dihydrofolate, 80 μM NADPH and the cell-free extract in a total volume of 2.5 ml. The reaction mixture without NADPH and dihydrofolate was preincubated for 3 min at 30°C, and then the reaction was initiated by adding NADPH and dihydrofolate. The decrease in absorbance at 340 nm for one min was measured with a Hitachi 124 Spectrophotometer.

Medium with added	Dihydrofolate reductase	
	Total activity* (nmol/min)	Specific activity* (nmol/min/mg protein)
Methotrexate	76	5.1
Biopterin	81	5.2

* The enzyme activity was defined as the amount of tetrahydrofolate produced per min under the standard assay conditions.

resistance toward MTX show an elevated activity of DFR.[6-9]

As shown in Table I, the DFR activity in the cells grown in the medium including 0.1 μM MTX was the same as that in the medium including 1.5 nM biopterin. As described in the following chapters (II and III), this protozoan has two types of DFR: A major DFR which reduces only dihydrofolate and is inhibited irreversibly by MTX and aminopterin at concentrations lower than 1 nM,[3] and PtR:DFR which reduces dihydrofolate and unconjugated pteridines such as L-*threo*-neopterin and 6-hydroxymethylpterin and is inhibited competitively by MTX (0.1 - 1 μM).[10] Small amounts of the PtR:DFR activity also existed in both lots of cells, when assayed with L-*threo*-neopterin as the substrate. This indicates that the types and amounts of DFR of *C. fasciculata* grown in MTX-medium were the same as those in the case of biopterin-medium.

Kidder *et al.*[11] have reported that *C. fasciculata* metabolizes folate to 6-hydroxymethylpterin and biopterin which are more effective growth factors. The amounts of MTX and aminopterin required for growth were the same as that of folate (230 - 2,300 nM).[12-15]

The present results indicate that this protozoan may metabolize MTX and aminopterin as well as folate to compounds that may be used effectively to promote growth.

REFERENCES

1) R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines," North-Holland Pub. Co., Amsterdam, London, 1969, p. 464.

2) H. N. Guttman, "Pteridine Chemistry," ed. by W. Pfleiderer and E. C. Taylor, Pergamon, London, 1964, p. 255.

3) K. Iwai, M. Kohashi and H. Oe, *Agric. Biol. Chem.*, 45, 113 (1981).

4) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193, 265 (1951).

5) H. Rembold and H. Metzger, *Hoppe-Seyler's Z. Phys. Chem.*, 329, 291 (1962).

6) G. A. Fischer, *Biochem. Pharmacol.*, 7, 75 (1961).

7) M. T. Hakala, S. F. Zakrewski and C. A. Nicol, *J. Biol. Chem.*, 236, 952 (1961).

8) M. Fiedkin, E. Crawford, S. R. Humphreys and A. Goldin, *Cancer Res.*, 22, 600 (1961).

9) R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines," North-Holland Pub. Co., Amsterdam, London, 1969, p. 130.

10) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 251(1983).

11) G. W. Kidder, V. C. Dewey and H. Rembold, *Arch. Mikrobiol.*, 59, 180 (1967).

12) H. A. Nathan and J. Cowperthwaite, *Proc. Soc. Exptl. Biol. Med.*,

85, 117 (1954).

13) H. A. Nathan, S. H. Hutner and H. L. Levin, *Nature*, 178,741(1956).

14) G. W. Kidder and B. N. Dutta, *J. Gen. Microbiol.*, 18, 621 (1958).

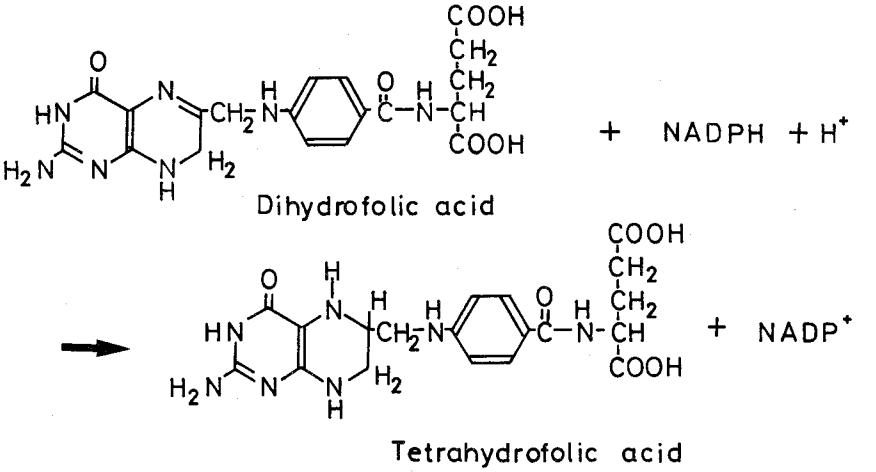
15) H. A. Nathan and H. B. Funk, *Am. J. Clin. Nutr.*, 7, 375 (1959).

a) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47,1651(1983).

Chapter II

Purification and Properties of Dihydrofolate Reductase from *Crithidia fasciculata* [b)]

As shown in Scheme I, an enzyme which catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate in the presence of NADPH (or NADH) has isolated from many sources.[1-6)] This enzyme, generally referred to as dihydrofolate reductase (DFR), bears the systematic name 5,6,7,8-tetrahydrofolate:NAD(P) oxidoreductase (EC 1.5.1.3). The enzyme also catalyzes the reduction of folate to tetrahydrofolate, but at a much slower rate than the reduction of dihydrofolate, particularly at physiological pH.[1,3)]



Scheme I

The occurrence of the DFR activity has been reported in high-speed centrifugal supernatant fractions of *C. fasciculata* ATCC 11745

and *Crithidia oncopelti*.[7] However, the detailed properties were not reported. This chapter deals with the purification and characteristic properties of DFR from *C. fasciculata* ATCC 12857.

MATERIALS AND METHODS

Materials. NADP, NADPH, NAD, NADH, bovine pancreas α-chymotrypsinogen *A*, hog stomach-mucosa pepsin, bovine liver glutamate dehydrogenase, ovalbumin, bovine serum albumin and aminopterin were purchased from Sigma Chemical Co. Pteroic acid and MTX were from Lederle Lab. Yeast extract was from Oriental Yeast Co. Polypepton was from Daigo-Eiyo Co. Hemin was from Tokyo-Kasei Kogyo Co. Liver fraction L was from Nutritional Biochemicals Co. γ-Globulins was from Mann Research Lab. Sephadex G-150, DEAE-Sephadex A-50, CM-Sephadex C-50 and Sepharose 4B were from Pharmacia Fine Chemicals. Other chemicals were purchased from Nakarai Chemicals Ltd., Kyoto. Dihydrofolate and dihydrohomofolate were prepared by the method of Futterman[8] and Kaufman,[9] respectively. Other pteridine compounds were reduced by the method of Shiota *et al.*[10]

Methods.

Growth of Crithidia fasciculata. *C. fasciculata* ATCC 12857 was grown by agitating at 25°C for 3 days in the culture medium described by Guttman[11] containing 0.5% (w/v) yeast extract, 0.5% (w/v) polypepton, 1.5% (w/v) sucrose, 0.0025% (w/v) hemin and 0.01% (w/v) liver fraction L. The cells were harvested by continuous centrifugation at 10,000 x *g* with a High Speed Centrifuge CM-60RN, Tomy

Seiki Co. Ltd., and then washed with saline. The moist cell paste was stored at -20°C until use to make extract.

Standard assay conditions for DFR. The DFR activity was assayed by the spectrophotometric method described by Mathews and Huennekens,[12] which is based on the decrease of absorbance at 340 nm due to conversions of NADPH to NADP and dihydrofolate to tetrahydrofolate. The molar extinction coefficient for reduction (12,300 $mol^{-1}cm^{-1}$ at 340 nm) was used to calculate the amount of dihydrofolate reduction.[13] The standard reaction mixture (2.5 ml) contained 50 mM potassium phosphate, pH 7.0, 30 mM 2-ME, 80 μM NADPH, 80 μM dihydrofolate and enzyme in a cuvette. The reaction mixture excepting NADPH and dihydrofolate was preincubated for 3 min at 30°C, and then the reaction was initiated by adding dihydrofolate and NADPH in this order at short intervals of 10 - 20 sec. A decrease in absorbance at 340 nm for one min was measured with a Hitachi 124 Spectrophotometer connected to a Hitachi QPE 34 recorder. The reaction proceeded linearly for 5 min. One unit of the enzyme activity is defined as the amount of enzyme which catalyzes the reduction of 1 μmol of dihydrofolate per min under the standard assay conditions.

Preparation of modified folate-Sepharose 4B. Folate-Sepharose 4B was prepared by modifying the method described by Whiteley *et al.*[14] Sepharose 4B was activated by CNBr according to a reported procedure.[14] The Sepharose was washed repeatedly with cold 0.1 M $NaHCO_3$ (pH 9) and then treated with 5 ml of hexamethyl-

enediamine (0.16 g) in the same buffer. After the mixture were stirred at 4°C for 24 hr, the product was collected by centrifugation and washed thoroughly with the buffer and water. An aqueous solution (5 ml) containing folic acid (56 mg/ml), adjusted to pH 6.5, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.15 g) was added to the above preparation of hexamethylenediamine-Sepharose resuspended in water (25 ml). After readjusting pH to 6.5, the mixture was stirred for 3 days at room temperature. The reacted gel was washed sequentially with water, 0.1 N NaOH and water. A small portion of the product gel (15 mg dry wt) was hydrolyzed with 6 N HCl (2 ml) at 110°C for 48 hr. From the amino acid analysis of glutamic acid in the supernatant solution of the hydrolyzed gel using a Hitachi KLA-5 analyzer, the amount of folate bound to Sepharose 4B was calculated to be 12 μmol of folate/g of dry gel.

Acrylamide gel electrophoresis. Acrylamide gel electrophoresis was performed at 2 mA per gel at pH 8.3 according to Davis.[15] SDS-polyacrylamide gel electrophoresis was conducted in 0.1% SDS-0.1 M phosphate buffer, pH 7.2 at 8 mA per gel according to Weber and Osborn.[16] To determine the position of protein, the gel was stained by incubating with 0.2% Coomassi brilliant blue G-250 for 5 hr. The gel was then destained in 10% methanol/10% acetic acid solution. The DFR activity on the gel was determined by the method according to Dunlap *et al.*[17]: The gel was incubated with 0.1 M Tris-HCl buffer (pH 7.0) containing 1 mM NADPH, 40 μM dihydrofolate and MTT (1 mg/ml) at room temperature for 30 min. The formation of purple band of

MTT-formazan was dependent upon tetrahydrofolate produced.

Gel-filtration for the determination of the molecular weight.

The molecular weight of DFR was estimated on a Sephadex G-150 column (1.5 x 84 cm) equilibrated with 0.1 M potassium phosphate, pH 6.5, containing 5 mM 2-ME and 1 mM EDTA. The column was calibrated with tryptophanase of *Proteus rettgeri* (220,000), bovine γ-globulin (160,000), bovine serum albumin (63,000), ovalbumin (43,000), pepsin (35,000) and α-chymotrypsinogen *A* (25,000) as markers of known molecular weight.

Determination of protein. Protein was determined by the method of Lowry *et al.*[18] using bovine serum albumin as the standard or by UV absorbance at 280 nm.

Preparation of purified DFR.

1) Preparation of homogenate. All operations were carried out at 0 - 4°C. Frozed cells (2 kg) of *C.fasciculata* were suspended in 6 volumes of 0.1 M potassium phosphate, pH 7.0, containing 1 mM EDTA. The cell suspension was disrupted by sonicating with a Kaijo-Denki 20 kHz Ultrasonic Oscillator and centrifuged at 12,000 x *g* for 20 min.

2) Ammonium sulfate fraction. Solid ammonium sulfate was added to the turbid supernatant to 35% of saturation. After stirring for 30 min, the precipitate was removed by centrifugation and discarded. The resulting supernatant was brought to 55% of saturation with additional ammonium sulfate.

3) DEAE-Sephadex A-50 column chromatography. The precipitate

was dialyzed against 40 liters of 25 mM potassium phosphate, pH 7.4, containing 1 mM EDTA. After removing insoluble materials by centrifugation, the supernatant (1,370 ml) was applied to a DEAE-Sephadex column (10 x 15 cm), equilibrated in advance with the same buffer. The column was developed with 1,500 ml of 25 mM potassium phosphate, pH 7.4, containing 1 mM EDTA and 0.3 M potassium chloride. The active fractions were pooled and brought to 55% of saturation with additional ammonium sulfate. The precipitate was dialyzed against 15 liters of 25 mM potassium phosphate, pH 7.4. The dialyzed solution (376 ml) was applied to the second DEAE-Sephadex column (2.5 x 38 cm). The column was developed by a linear gradient of potassium chloride from 0 to 0.3 M in 25 mM potassium phosphate, and fractions of 20 ml each were collected. The active fractions were pooled and precipitated by adding solid ammonium sulfate to 55% saturation. The precipitate was dialyzed against 50 mM potassium phosphate, pH 7.4.

4) Sephadex G-150 column chromatography. The dialyzed solution was subjected to gel filtration on a Sephadex G-150 column (6 x 72 cm), equilibrated in advance with 50 mM potassium phosphate, pH 7.4. Fractions of 20 ml each were collected. The fractions between #38 and #48 (fraction I), #49 and #60 (fraction II) were pooled. The major fraction II containing high enzyme activity was precipitated by adding solid ammonium sulfate to 55% of saturation. The precipitate was dialyzed against 10 liters of 50 mM potassium phosphate, pH 6.5, containing 1 mM EDTA and 5 mM 2-ME.

5) CM-Sephadex C-50 column chromatography. The dialyzed solution of fraction II was applied to a CM-Sephadex column (2.5 x 37 cm), equilibrated in advance with the same buffer as described above. After being washed with the same buffer, the column was developed with a linear gradient of 300 ml of 50 mM potassium phosphate, pH 6.5, containing 1 mM EDTA and 5 mM 2-ME in the mixing chamber and 300 ml of 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA, 5 mM 2-ME and 0.5 M potassium chloride in the reservoir. Fractions of 10 ml each were collected. Each fraction between #6 and #13 (fraction IIb), #47 and #55 (fraction IIa), and #59 and #65 (minor fraction) was pooled. After being concentrated with a collodion bag, fraction IIa was dialyzed against 5 liters of 50 mM potassium phosphate, pH 6.5, containing 1 mM EDTA and 8 mM 2-ME.

6) 2nd Sephadex G-150 column chromatography. The dialyzed solution was applied to a Sephadex G-150 column (1.5 x 90 cm), equilibrated in advance with the same buffer. Fractions of 2 ml each were collected.

7) Affinity chromatography on folate-Sepharose 4B. The active fraction was applied to a folate-Sepharose 4B column (0.9 x 5.5 cm), equilibrated in advance with 50 mM potassium phosphate, pH 6.5, containing 1 mM EDTA and 8 mM 2-ME. The column was eluted by 30 ml of the above buffer containing 0.5 M potassium chloride, and then by 20 ml of the buffer containing 0.5 M potassium chloride and 1 mM dihydrofolate. The active fractions eluted by the latter buffer were collected and dialyzed against the above buffer containing 0.5 M

potassium chloride. About 80% of the DFR activity was recovered by this column procedure.

RESULTS

Purification of DFR The elution profiles for Sephadex G-150 and CM-Sephadex chromatographies are presented in Fig. 1. Table I summarizes the purification procedure for DFR IIa from 2 kg of *Crithidia* cells. The enzyme was purified 2744-fold with a yield of 5.3%. The final preparation had a specific activity of 6860 and was homogeneous as shown by disc gel electrophoresis (Fig. 2). Only one protein band (gel A) was obtained and its position was cor-

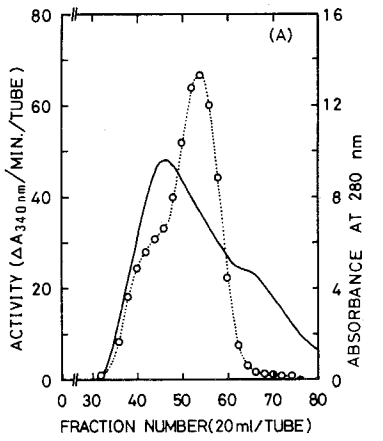


FIG. 1A. Chromatography on Sephadex G-150 of Dihydrofolate Reductase of *Crithidia fasciculata*.

The reductase fraction obtained by a 2nd DEAE-Sephadex eluate was applied to a column (6 × 72 cm) of Sephadex G-150, equilibrated with 50 mM potassium phosphate, pH 7.4. Each fraction of #38 to 48 (reductase fraction I), and #49 to 60 (reductase fraction II) was pooled.

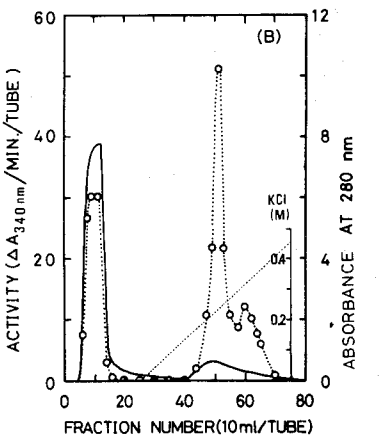


FIG. 1B. Chromatography on CM-Sephadex of the Reductase Fraction II.

The reductase fraction II obtained by a Sephadex G-150 eluate was applied to a column (2.5 × 37 cm) of CM-Sephadex C-50, equilibrated with the same buffer as seen in Fig. 1A. The column was developed with a linear gradient using 300 ml of 50 mM potassium phosphate at pH 6.5/1 mM EDTA and 5 mM 2-mercaptoethanol in the mixing chamber and 300 ml of the same buffer at pH 7.5/0.5 M KCl in the reservoir. Each fraction of #6 to 13 (reductase fraction IIb), and #47 to 55 (reductase fraction IIa) was pooled.

TABLE I. SUMMARY OF THE PURIFICATION OF DIHYDROFOLATE REDUCTASE IIa FROM *Crithidia fasciculata*

Purification step	Protein (mg)	Total activity (units*)	Specific activity (units* $\times 10^3$/mg of protein)	Yield (%)	Purification (fold)
Homogenate	179,253	456	2.5	100.0	1
Ammonium sulfate 35～55% saturation	30,414	398	13.1	87.2	5
DEAE-Sephadex	15,072	336	22.3	73.7	9
2nd DEAE-Sephadex	5,845	217	37.1	47.6	15
Sephadex G-150	1,610	125	77.4	27.3	31
CM-Sephadex	61	54	893	11.9	357
2nd Sephadex G-150	24	30	1248	6.6	499
Folate-Sepharose 4B	3.5	24	6860	5.3	2744

* One unit of the enzyme activity is defined as the amount of enzyme which catalyzes the reduction of 1 μmol of dihydrofolate per min under the standard assay conditions.

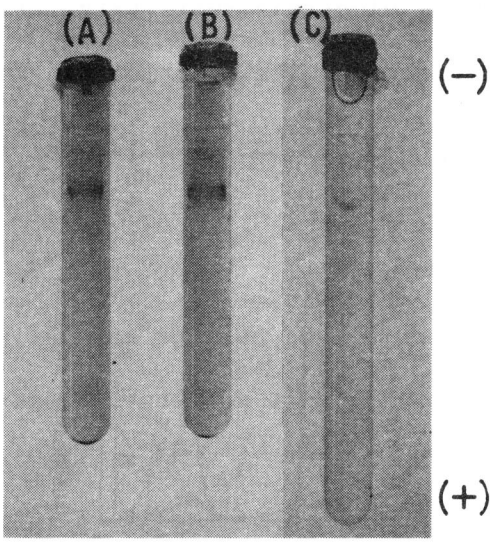


FIG. 2. Electrophoresis on Polyacrylamide Gel of the Purified Dihydrofolate Reductase IIa (A, B) and the SDS-treated Enzyme (C).
Gels A and C were stained with Coomassie brilliant blue G-250 for protein. Gel B was incubated with a dye (see text) to demonstrate enzyme activity.

responded to a band containing enzyme activity (gel B).

After being treated with 2-ME, the fraction I was eluted at a similar fraction to the reductase IIa on a Sephadex G-150 column. Fraction I could not be obtained by chromatography of the homogenate on a Sephadex G-150 column. Thus, fraction I was estimated to be a polymer of the reductase IIa.

The physical properties of fraction IIb was similar to the reductase IIa and had ability to reduce several unconjugated pterins as well as dihydrofolate (see Chapters III and IV).

Stability of the enzyme The purified preparation was stabi-

lized by the presence of 4 mM 2-ME and 30% glycerol, although the inclusion of 2-ME in the purification steps, especially in the ammonium sulfate fraction, lost totally the DFR activity.

Molecular weight and subunit structure As seen in Fig. 3 (upper panel), a molecular weight of 110,000 daltons was estimated by gel filtration on a Sephadex G-150 column. As seen in Fig. 2-C, SDS-treated reductase IIa revealed a single polypeptide band. As seen in Fig. 3 (lower panel), the molecular weight of the SDS-treated reductase IIa was estimated to be 58,000 daltons. These results indicate that DFR IIa consists of 2 subunits with the same molecular weight. Thus, a molar activity of 755 mol of tetrahydrofolate produced per min per mol of the enzyme was calculated from the amount of dihydrofolate reduced using a molar extinction coefficient for the DFR reaction (12,300 $mol^{-1} cm^{-1}$).[13]

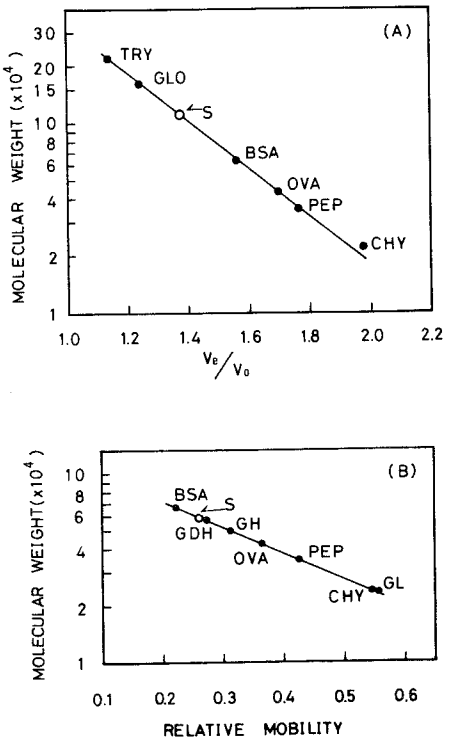


FIG. 3. Estimation of Molecular Weight of Dihydrofolate Reductase IIa by Sephadex G-150 (Upper Panel) and by Electrophoresis on SDS Polyacrylamide Gel (Lower Panel).

The Sephadex column was calibrated with: TRY, tryptophanase; GLO, γ-globulins; BSA, bovine serum albumin; OVA, ovalbumin; PEP, pepsin, and CHY, α-chymotrypsinogen A. Elution volume *versus* void volume (*Ve*/*Vo*) was plotted against molecular weight. The marker proteins used for electrophoresis were BSA, OVA, PEP, CHY, GDH (glutamic dehydrogenase), GH (H-chain of γ-globulins), and GL (L-chain of γ-globulins). Each mobility *versus* BPB mobility (relative mobility) was plotted against molecular weight. S (upper panel), dihydrofolate reductase IIa; S (lower panel), SDS-treated reductase IIa.

pH optimum As seen in Fig. 4, the reductase IIa shows only a single pH optimum at 7.0.

Substrate and cofactor specificities The purified DFR IIa had the absolute requirement for dihydrofolate and NADPH. In the presence of NADPH, dihydrohomofolate (80 μM) was slightly effective as substrate at 1% dihydrofolate, but it was ineffective as substrate in the presence of NADH. The following pteridine compounds were ineffective as substrates at 80 μM in the presence of 80 μM of NADPH or NADH at pH values from 4 to 9: Folate, 10-formyldihydrofolate, pteroate, 7,8-dihydropteroate, L-*erythro*-biopterin, L-*erythro*-7,8-dihydrobiopterin, L-*threo*-neopterin, L-*threo*-7,8-dihydroneopterin.

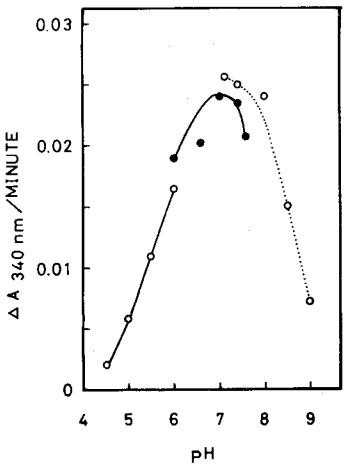


FIG. 4. Effect of pH on the Activity of Dihydrofolate Reductase IIa.
○—○, sodium citrate buffer; ●—●, potassium phosphate buffer; ○---○, Tris-HCl buffer.

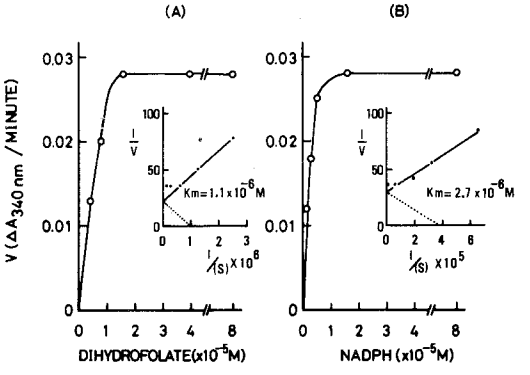


FIG. 5. Effect of Dihydrofolate (A) and NADPH (B) on the Activity of Dihydrofolate Reductase IIa.
The inset shows Lineweaver–Burk plots.

The reaction product of dihydrofolate was identified as tetrahydrofolate by a reported spectrophotometric method using NADPH-regenerating system of glucose-6-phosphate dehydrogenase.[19] The reverse reaction was obtained

at only one tenth of the forward reaction.

Kinetic parameters The apparent *Km* values were calculated from a double reciprocal plots. As seen in Fig. 5, the *Km* values for dihydrofolate and NADPH were 1.1 and 2.7 μM, respectively. As shown in Table II, 10-formyltetrahydrofolate, 5-formyltetrahydrofolate, folate, 5,10-methenyltetrahydrofolate, 5-methyltetrahydrofolate, tetrahydrofolate and 5,10-methylenetetrahydrofolate inhibited the reductase IIa activity in this order. The inhibition by each folate compound was competitive toward dihydrofolate, and the *Ki* values for 10-formyltetrahydrofolate, 5-formyltetrahydrofolate, folate and tetrahydrofolate (as estimated from Dixon plots[20]) were 0.64, 5.2, 16 and 130 μM, respectively. 10-Formyldihydrofolate also inhibited competitively, and its *Ki* value was 1.4 μM. Antifolates such as MTX, aminopterin, pyrimethamine and trimethoprim inhibited the enzyme activity. The inhibition by the latter two

Table II. Effect of Tetrahydrofolates and Folate on Dihydrofolate Reductase IIa

The enzyme was preincubated with the folate compound for 3 min at 30°C, and then the reaction was initiated by adding 80 μM of NADPH and 80 μM of dihydrofolate in this order. The enzyme activity is expressed as relative activity (%) to the untreated enzyme.

Addition	Concentration (μM)	Relative activity (%)
None	-	100.0
10-Formyltetrahydrofolate	8	36.3
5-Formyltetrahydrofolate	50	32.4
5,10-Methenyltetrahydrofolate	80	55.7
5,10-Methylenetetrahydrofolate*	80	97.7
5-Methyltetrahydrofolate	74	87.5
Tetrahydrofolate	74	96.3
Folate	80	37.5

* The enzyme activity was assayed in 50 mM potassium maleate buffer, pH 7.0.

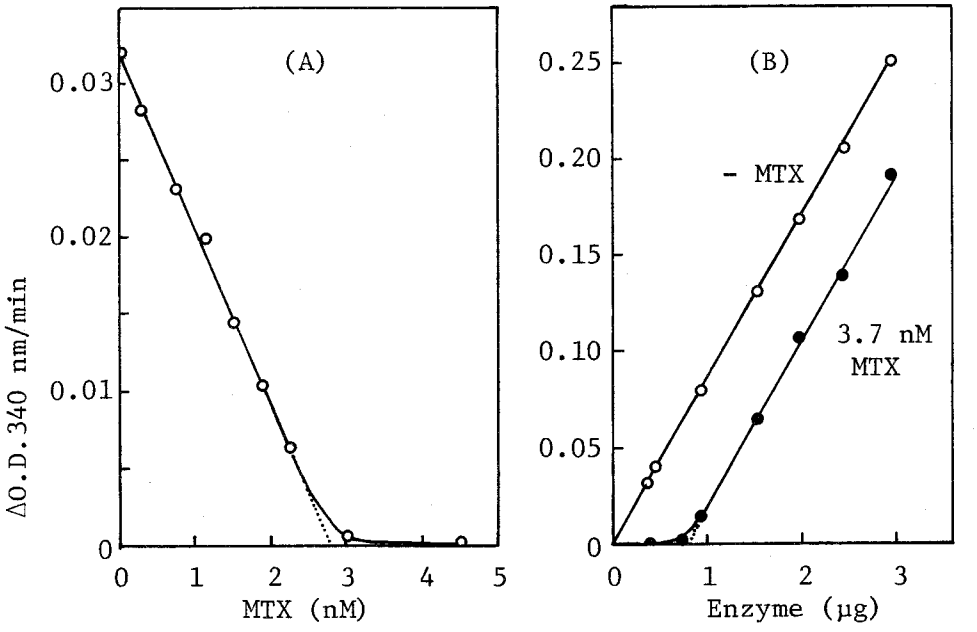


Fig. 6. Titration of the Purified Dihydrofolate Reductase IIa by MTX.

(A): The purified DFR IIa (3.4 nM) was preincubated for 3 min at 30°C with varying amounts of MTX in the presence of 80 μM NADPH, and the reaction was initiated by adding 80 μM dihydrofolate.

(B): The varying amounts of the enzyme were preincubated with (●) or without (○) 3.7 nM MTX.

compounds was also competitive toward dihydrofolate, and the *Ki* values for pyrimethamine and trimethoprim were 0.3 and 1.5 μM, respectively. On the contrary, MTX and aminopterin showed a stoichiometric inhibition as reported on many DFRs.[3] Figure 6 represents the interaction of MTX with the purified DFR IIa either by titration of a fixed amount of the enzyme with MTX (A) or titration of a fixed amount of MTX with the enzyme (B). The intercept at the abscissa indicates the concentration of MTX required to inhibit quantitatively an amount of the enzyme. From (A) and (B) in Fig. 6, it was calculated that one mol of the enzyme was inhibited by 0.81 and 0.86 mol of MTX, respectively. The concentration of

aminopterin required to cause complete inhibition of one mol of the enzyme was also estimated to be 0.88 mol.

NADP inhibited the enzyme activity. The inhibition by NADP was competitive toward NADPH, and its *Ki* value was 8 μM.

Effect of sulfhydryl reagents, chaotropic agents, urea and metal ions As shown in Table III, the enzyme activity was inhibited strongly by 1.2 μM of pCMB and 0.2 mM of NEM. The inhibition was recovered by adding 8 mM of 2-ME. Urea and chaotropic agents such as guanidine-HCl and formamide also inhibited the enzyme activity. The enzyme denatured with 4 M of urea, and could not be renatured by removing urea by dialysis. Less than 0.1 M of K^+ had no effect on the activity. More than 0.2 M of K^+, however, inhibited the activity. Other monovalent cations (Na^+, NH_4^+, Li^+ and Cs^+) or divalent cations (Mg^{2+}, Ba^{2+} and Ca^{2+}) had no effect.

TABLE III. EFFECT OF SULFHYDRYL REAGENTS, CHAOTROPIC AGENTS, AND UREA ON DIHYDROFOLATE REDUCTASE IIa

After the enzyme was preincubated with the reagent for 5 min at 30°C, the enzyme activity was measured under standard assay conditions. The enzyme activity is expressed as relative activity (%) to the untreated enzyme.

Addition		Relative activity (%)
None		100.0
pCMB*	1.2 μM	53.1
	1.2 μM+2-ME** (8 mM)	78.5
NEM***	0.2 mM	20.0
	0.2 mM+2-ME** (8 mM)	91.4
Guanidine-HCl	0.08 M	96.0
	0.2 M	70.0
Formamide	0.8 M	86.2
	1.6 M	66.7
Urea	2.0 M	56.5
	4.0 M	7.0

* *p*-Chloromercuribenzoate.
** 2-Mercaptoethanol.
*** *N*-Ethylmaleimide.

DISCUSSION

Three different fractions (I, IIa and IIb) having the activity

of DFR were obtained from the cell-free extract of *C. fasciculata* ATCC 12857. A major fraction IIa was homogeneously isolated and its properties were characterized. A molecular weight of DFR isolated from the most organisms has been estimated to be 15,000 to 30,000 daltons.[1-3] But, the molecular weight of the purified DFR IIa (110,000 daltons) was similar to those of DFRs from *Trypanosoma* sp.,[21] *Crithidia oncopelti*,[21] *Plasmodium* sp.[22,23] and soybean seedlings,[5] which molecular weights have been reported to be 100,000 to 200,000 daltons. The *Km* values for dihydrofolate (1.1 μM) and NADPH (2.7 μM) for the DFR IIa were similar to those of the four DFRs described above. Like the *Plasmodium* sp.,[22] *C. fasciculata* ATCC 11745,[7] *C. oncopelti*,[7] and soybean[5] DFRs, the DFR IIa was unable to use folate as a substrate. In the genus *Crithidia*, NADH did not replace NADPH as a hydrogen donor. Several DFRs have been activated by pCMB,[24-27] guanidine-HCl,[26,27] urea,[23,25-30] and monovalent or divalent cations,[22,23,26,27,29-31] whereas the DFR IIa activity was inhibited by these compounds. This property of the enzyme is characteristic in *C. fasciculata* ATCC 12857, and could not be observed in all other DFRs isolated thus far. The pH optimum of the DFR IIa was 7.0, similar to the ranges found for *C. fasciculata* ATCC 11745,[7] *C. oncopelti*,[7] soybean[5] and *Plasmodium* sp.[22]

Naturally occurring folates such as folate,[27,32] 10-formylpteroylmono-[27,30,33] or penta-glutamates[34] and tetrahydrofolate derivatives[29] including 5,10-methylene, 5,10-methenyl, 10-formyl and 5-formyltetrahydrofolates are relatively strong inhibitors of

DFR. The DFR IIa activity was strongly inhibited by folate and tetrahydrofolates (Table II). The DFR IIa was 38 and 23 times more sensitive to inhibition by 10-formyltetrahydrofolate and 5-formyltetrahydrofolate than the bovine liver DFR,[29] respectively. NADP, one of the reaction products of DFR, has also been shown to cause inhibition of many DFRs.[3] The DFR IIa was 16 times more sensitive to NADP than tetrahydrofolate, based on comparison with the *Ki* values. These evidences suggest that the *Crithidia* DFR may be regulated by levels of tetrahydrofolates and NADP *in vivo*.

MTX and aminopterin were stoichiometric inhibitors of the DFR IIa as in the case of a number of other DFRs.[1-3] The data of the MTX titration indicate that one molecule of the DFR IIa consisting of two subunits with the same molecular weight (58,000) may have a single binding site for MTX (or aminopterin).

Gutteridge *et al.*[7] have classified DFRs to three groups of mammalian, trypanosomal and bacterial DFRs by measuring the concentration of trimethoprim required to effect a 50% decrease in the enzyme activity. A 50% inhibitory concentration of trimethoprim for the DFR IIa was 27 μM. Thus, the DFR IIa in *C. fasciculata* ATCC 12857 was only one tenth as sensitive to the inhibition as was the enzyme in *C. fasciculata* ATCC 11745.[7] The relative insensitivity of the DFR IIa to trimethoprim was rather similar to that of *C. oncopelti* and man DFRs.[7]

Multiple forms of DFR have isolated from bacteria such as *Lactobacillus casei*,[1,35] *Streptococcus faecium* var *durans*,[36,37]

Esherichia coli B (RT 500)[38,39] and *Diplococcus pneumoniae*,[40] protozoa such as *Trypanosoma* sp.[21] and *C. oncopelti*,[21] and mammalian sources such as chicken liver,[1] bovine liver,[41,42] hamster kidney cells[43] and L1210 lymphoma cells.[44] The present data indicate that there may be at least four DFR fractions (I, IIa, IIb and minor) in *C. fasciculata* ATCC 12857. Like the bovine liver,[41,42] hamster kidney,[43] *E. coli* B[38,39] and *D. pneumoniae*[40] DFRs, fraction I was a polymeric form of the DFR IIa. Minor fraction appeared to be the DFR IIa bound with NADPH as seen in the chicken liver,[1,45,46] L1210 cells[44] and *L. casei*[1,35] DFRs. Fraction IIb has similar physical properties to the purified DFR IIa. However, fraction IIb was able to reduce dihydropteroate and several oxidized unconjugated pteridines at a similar level with dihydrofolate, while the DFR IIa reduced specifically dihydrofolate (see Chapters III and IV).

C. fasciculata has a nutritional requirement for folate as well as biopterin.[47] But, any evidence of folate reduction was unable to detected by the present study either with four DFR fractions or the crude extract of *C. fasciculata* in the presence of NADPH (or NADH). A small part of folate has been reported to be converted to 6-hydroxymethylpterin and biopterin in *C. fasciculata*.[48] The auther has found that MTX and aminopterin promotes the growth of *C. fasciculata* at the same level as folate.[49] These evidences suggest that *C. fasciculata* may have the metabolic pathways for folate and anti-folates such as MTX and aminopterin.

REFERENCES

1) F. M. Huennekens, R. B. Dunlap, J. H. Freisheim, L. E. Gundersen, N. G. L. Harding, S. A. Levison and G. P. Mell, *Annal. N. Y. Acad. Sci.*, 186, 85 (1971).

2) F. M. Huennekens, "Biological Oxidations," ed. by T. P. Singer, Interscience, New York, 1968, p. 439.

3) R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines," North-Holland Pub. Co., Amsterdam, London, 1969, p.139.

4) N. Suzuki and K. Iwai, *Plant & Cell Physiol.*, 11, 199 (1970).

5) V. A. Reddy and N. A. Rao, *Arch. Biochem. Biophys.*, 174,675(1976).

6) M. M. Cymborowska and Z. M. Zielinska, *Comp. Biochem. Physiol.*, 50B, 35 (1975).

7) W. E. Gutteridge, J. J. McCormack and J. J. Jaffe, *Biochim. Biophys. Acta*, 178, 453 (1969).

8) S. Futterman, *J. Biol. Chem.*, 228, 1031 (1957).

9) S. Kaufman, *J. Biol. Chem.*, 242, 3934 (1967).

10) T. Shiota, M. N. Disraely and M. P. McCann, *J. Biol. Chem.*, 239, 2259 (1964).

11) H. N. Guttman, "Pteridine Chemistry," ed. by W. Pfleiderer and E. C. Taylor, Pergamon Press, London, 1964, p. 255.

12) C. K. Mathews and F. M. Huennekens, *J. Biol. Chem.*, 238,3436(1963).

13) B. L. Hillcoat, P. F. Nixon and R. L. Blakley, *Anal. Biochem.*, 21, 178 (1967).

14) J. M. Whiteley, R. C. Jacson, G. P. Mell, J. H. Drais and F. M. Huennekens, *Arch. Biochem. Biophys.*, 150, 15 (1972).

15) B. J. Davis, *Annal. N. Y. Acad. Sci.*, 121, 404 (1964).

16) K. Weber and M. Osborn, *J. Biol. Chem.*, 244, 4406 (1969).

17) R. B. Dunlap, L. E. Gundersen and F. M. Huennekens, *Biochem. Biophys. Res. Commun.*, 42, 772 (1971).

18) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193, 265 (1951).

19) M. J. Osborn and F. M. Huennekens, *J. Biol. Chem.*, 233, 969 (1958).

20) M. Dixon, *Biochem. J.*, 55, 170 (1953).

21) W. E. Gutteridge, J. J. Jaffe and J. J. McCormack, *Biochim. Biophys. Acta*, 191, 753 (1969).

22) E. G. Platzer, *J. Protozool.*, 21, 400 (1974).

23) R. Ferone, J. J. Burchall and G. H. Hichings, *Mol. Pharmacol.*, 5, 49 (1969).

24) B. T. Kaufman, *J. Biol. Chem.*, 239, 669 (1964).

25) J. Perkins and J. R. Bertino, *Biochem. Biophys. Res. Commun.*, 15, 121 (1964).

26) J. L. McCullough and J. R. Bertino, *Biochem. Pharmacol.*, 20, 561 (1971).

27) J. Perkins and J. R. Bertino, *Biochemistry*, 4, 847 (1965).

28) B. T. Kaufman, *Biochem. Biophys. Res. Commun.*, 10, 449 (1963).

29) P. B. Rowe and P. J. Russel, *J. Biol. Chem.*, 248, 984 (1973).

30) S. J. Johnson and S. V. Gupta, *Can. J. Biochem.*, 60, 1132 (1982).

31) J. R. Bertino, *Biochim. Biophys. Acta*, 58, 377 (1962).

32) D. R. Morales and D. M. Greenberg, *Biochim. Biophys. Acta*, 85, 360 (1964).

33) M. d'Urso-Scott, J. Uhoch and J. R. Bertino, *Proc. Nat. Acad. Sci. USA*, 71, 2736 (1974).

34) M. Friedkin, L. T. Plante, E. J. Crawford and M. Crumm, *J. Biol. Chem.*, 250, 5614 (1975).

35) L. E. Gundersen, R. B. Dunlap, N. G. L. Harding, J. H. Freisheim, F. Otting and F. M. Huennekens, *Biochemistry*, 11, 1018 (1972).

36) A. M. Albrecht, F. K. Pearce, W. J. Suling and D. J. Hutchison, *Biochemistry*, 8, 960 (1969).

37) P. F. Nixon and R. L. Blakley, *J. Biol. Chem.*, 243, 4722 (1968).

38) D. P. Baccanari, A. Phillips, S. Smith, D. Sinski and J. Burchall, *Biochemistry*, 14, 5267 (1975).

39) D. P. Baccanari, D. Averett, C. Briggs and J. Burchall, *Biochemistry*, 16, 3566 (1977).

40) F. M. Sirotonak and W. A. Williams, *Arch. Biochem. Biophys.*, 136, 580 (1970).

41) H. Baumann and K. J. Wilson, *Eur. J. Biochem.*, 60, 9 (1975).

42) B. T. Kaufman and V. F. Kemerer, *Arch. Biochem. Biophys.*, 172, 289 (1976).

43) U. J. Hänggi and J. W. Littlefield, *J. Biol. Chem.*, 249, 1390 (1974).

44) J. P. Perkins, B. L. Hillcoat and J. R. Bertino, *J. Biol. Chem.*, 242, 4771 (1967).

45) G. P. Mell, M. Martelli, J. Kirchner and F. M. Huennekens, *Biochem. Biophys. Res. Commun.*, 33, 74 (1968).

46) F. M. Huennekens, G. P. Mell, N. G. L. Harding, L. E. Gundersen and J. H. Freisheim, "Chemistry and Biology of Pteridines," ed.

K. Iwai, M. Goto, M. Akino and Y. Iwanami, International Academic Print. Co., Tokyo, 1970, p. 329.

47) G. W. Kidder and B. N. Dutta, *J. Gen. Microbiol.*, 18, 621 (1958).

48) G. W. Kidder, V. C. Dewey and H. Rembold, *Arch. Mikrobiol.*, 59, 180 (1967).

49) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 1651 (1983).

b) The major part of this chapter has been published in [*Agric. Biol. Chem.*, 45, 113 (1981)].

Chapter III

The Occurrence and Properties of Pteridine Reductase: Dihydrofolate Reductase in *Crithidia fasciculata*[c)]

In Chapter II, the author has isolated from *C. fasciculata* three fractions (I, IIa and IIb) having DFR activity, and fraction IIa, a major DFR, has been isolated homogeneously.[1)] DFR has been isolated from various sources,[2-4)] including protozoa such as *Plasmodium*,[5,6)] *Crithidia*[1,7-9)] and *Trypanosoma*.[7)] Some DFRs also reduce folate[2-4)] and some dihydropteridine compounds,[10-12)] as well as dihydrofolate. Fraction IIa, as well as DFRs from protozoa [1,5,6,8)] specifically reduced dihydrofolate. This suggests that fractions I and IIb may have some different substrate specificities from fraction IIa. In the present chapter, fraction IIb was further purified, and some of its properties were characterized. The data showed that fraction IIb reduced various oxidized forms of pteridine compounds, as well as dihydrofolate and dihydropteroate. This property renders this enzyme quite different from any other DFR isolated so far. Fraction IIb still had a dihydrofolate-reducing activity, so the auther named it pteridine reductase:dihydrofolate reductase (PtR:DFR).

MATERIALS AND METHODS

Materials. Pterin and MTT were obtained from Sigma Chemical Co. Glucose-6-phosphate was from Calbiochem. Yeast glu-

cose-6-phosphate dehydrogenase (140 units/mg of protein) was from Boehringer Mannheim GmbH. Other materials and chemicals were as described in Chapter II. The dihydro- or tetrahydro-forms of the pteridine compounds were reduced according to the procedure described by Kaufman.[12]

Methods.

Purification of fraction IIb (pteridine reductase:dihydrofolate reductase). All operations were carried out at 0 - 4°C. Fractions between #6 and #13 collected from a CM-Sephadex column, as described in Chapter II, were pooled, identified as fraction IIb and used as the starting material for purifying pteridine reductase:dihydrofolate reductase (PtR:DFR).

a) *Ammonium sulfate precipitation.* The protein in fraction IIb (80 ml) was precipitated by adding solid ammonium sulfate to give 55% saturation. After stirring for 30 min, the precipitate was collected by centrifugation at 10,000 x *g* for 10 min and dialyzed against 10 liters of 50 mM potassium phosphate, pH 7.4, containing 1 mM EDTA and 8 mM 2-ME.

b) *DEAE-Sephadex A-50 column chromatography.* The dialyzed solution (15 ml) was applied to a DEAE-Sephadex column (2.5 x 20 cm), equilibrated in advance with the same buffer. The column was eluted by a linear salt gradient with 200 ml of 50 mM potassium phosphate, pH 7.4, in the mixing chamber and 200 ml of the same buffer supplemented with 0.25 M potassium chloride in the reservoir. Five milliliter fractions were collected. The active fractions

were pooled and concentrated to 2ml by a collodion bag.

c) *Sephadex G-150 column chromatography.* The concentrated solution was applied to a Sephadex G-150 column (1.5 x 85 cm), equilibrated in advance with 50 mM potassium phosphate, pH 7.4, containing 1 mM EDTA and 8 mM 2-ME. Two milliliter fractions were collected, and the active fractions were pooled and concentrated to 2 ml (150 mg protein). This final solution was used as the enzyme source.

Standard assay conditions for pteridine reductase:dihydrofolate reductase. Pteridine reductase activity was measured by a photometric method, based on a decreased in absorbance at 340 nm due to contributions from the oxidation of NADPH to NADP and the reduction of the pteridine compound to a tetrahydropteridine compound. Since it was determined that two mol of NADPH consumed during the enzymic reduction of one mol of L-*threo*-neopterin (Fig. 3), this reaction was formulated as follows:

$$\text{L-}threo\text{-Neopterin} + 2\text{NADPH} + 2\text{H}^{+} \longrightarrow \text{L-}threo\text{-Tetrahydroneopterin} + 2\text{NADP}^{+}$$

In this reaction the difference in molar extinction coefficients ($\Delta\varepsilon$) at 340 nm was equal to the sum of 2 x 6,150 $mol^{-1}cm^{-1}$ (twice the $\Delta\varepsilon$ for the NADPH change at pH 6.0) plus 5,000 $mol^{-1}cm^{-1}$ (the $\Delta\varepsilon$ for L-*threo*-neopterin reduction), or 17,300 $mol^{-1}cm^{-1}$. The molar extinction differences ($\Delta\varepsilon$'s) for the enzymic reduction of 6-methylpterin and 6-hydroxymethylpterin were also calculated as 18,000 and 17,600 $mol^{-1}cm^{-1}$, respectively.

The standard reaction mixture, in a total volume of 2.5 ml, contained 50 mM sodium citrate, pH 6.0, 30 mM 2-ME, 60 - 80 μM pteridine compound, 80 μM NADPH and enzyme solution (90 μg of protein). The reaction mixture, without NADPH, was preincubated for 3 min at 30°C, then the reaction was initiated by the addition of NADPH. The decrease in absorbance at 340 nm for one min was measured with a Hitachi 124 Spectrophotometer. (The reaction with each pteridine compound proceeded linearly for 5 min.) The activity, with the pteridine compounds as substrates, is defined as absorbancy decrease at 340 nm for one min.

However, the enzyme activity, using xanthopterin as a substrate, was measured at 390 nm under the standard assay conditions in the absence of 30 mM 2-ME, since the xanthopterin (λmax 275 and 387 nm) reacted rapidly with 30 mM 2-ME in 50 mM sodium citrate at pH 6.0 and changed to an unknown compound [λmax 286 and 310 nm (inflection)].

DFR activity was measured by the method described in Chapter II.[1)]

Assay conditions for sepiapterin reductase. Sepiapterin reductase activity was determined by the method described by Matsubara *et al.*[13)]

UV spectrum of reaction product. That the PtR:DFR-catalyzing reaction with pteridine compounds can be followed spectrophotometrically with the aid of a NADPH-regenerating system has been demonstrated by Osborn and Huennekens.[14)] The reaction mixture, in a total volume of 2.5 ml, contained 50 mM potassium phosphate, pH 7.0,

30 mM 2-ME, 20 - 60 μM pteridine compound, 20 μM NADPH, 10 mM glucose-6-phosphate, glucose-6-phosphate dehydrogenase (2.8 units) and enzyme solution (360 μg of protein). The reaction mixture without the pteridine compound was preincubated at 30°C for 30 min, and the reaction was initiated by the addition of the pteridine compound. The spectral changes occurring between 400 and 240 nm were measured at 6 min intervals by a Shimazu Multipurpose Recording Spectrophotometer MPS-5000 with a recording speed of 400 nm per min.

Polyacrylamide gel electrophoresis. Electrophoresis on polyacrylamide gel was performed under the same conditions as described in Chapter II.[1] The activity for PtR:DFR on the gel was also determined by the method already described.[1]

Determination of protein concentration. Protein concentration was determined by the method of Lowry *et al.*[15] using bovine serum albumin as the standard.

RESULTS

Purification of pteridine reductase:dihydrofolate reductase

The final preparation of fraction IIb had both PtR and DFR activities. The DFR activity was purified 60-fold with a yield of 5% from the crude homogenate,[1] while this same procedure yielded a 600-fold of the PtR activity when L-*threo*-neopterin was used as the substrate. This enzyme contaminated one minor protein band obtained on disc polyacrylamide gel electrophoresis (Fig. 1,C). When the gel was incubated further in the presence of 6-hydroxy-

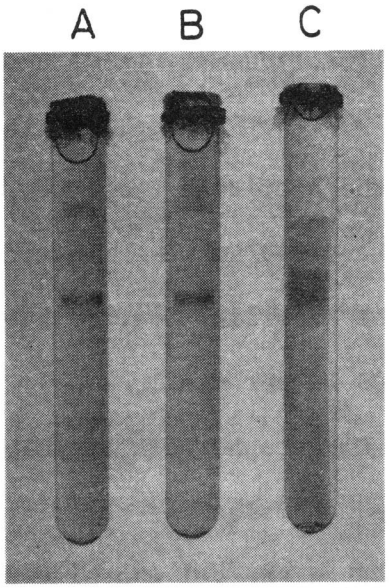


FIG. 1. Electrophoresis on Polyacrylamide Gel of Pteridine Reductase : Dihydrofolate Reductase.

Gels A and B were stained with MTT for 30 min at room temperature in the presence of 40 μM of 6-hydroxymethylpterin and dihydrofolate, respectively. Gel C was stained for protein with Coomassie brilliant blue G-250.

methylpterin (Fig. 1,A) or dihydrofolate (Fig. 1,B), two active bands appeared at the same position on the gels. This indicates that the main protein band had both PtR and DFR activities. The enzyme had a specific activity of 154 nmol dihydrofolate reduction per min per mg of protein at pH 6.8. However, considering the results obtained from the NADPH-consumption studies described later in this chapter, the enzyme reduced 65, 62 and 75 nmol of 6-hydroxymethylpterin, 6-methylpterin and L-*threo*-neopterin, respectively, at pH 6.0.

Stability of the enzyme The enzyme was stabilized in the presence of ammonium sulfate. The precipitate obtained from 55% saturation retained both PtR and DFR activities at 0°C for a minimum of one year.

Molecular weight of the enzyme The molecular weight of the enzyme was estimated to be 110,000 daltons by Sephadex G-150 gel filtration using the same procedure described in Chapter II.[1)]

Substrate specificity and pH optimum As shown in Table I,

TABLE I. SUBSTRATE SPECIFICITY OF PTERIDINE REDUCTASE: DIHYDROFOLATE REDUCTASE FOR CONJUGATED AND UNCONJUGATED PTERIDINE COMPOUNDS

Compound	Enzyme activity[a] ($\Delta A_{340\,nm}$/min)
Dihydrofolate	0.068 (6.8)[b]
Folate	0 (4.5～9.0)
Dihydropteroate	0.055 (7.0)
Pteroate	0.001 (7.0)
L-*threo*-7,8-Dihydroneopterin	0.015 (6.0)
L-*threo*-Neopterin	0.047 (6.0)
L-*erythro*-Neopterin	0.020 (6.0)
D-*threo*-Neopterin	0.019 (6.0)
D-*erythro*-Neopterin	0.013 (6.0)
L-*erythro*-7,8-Dihydrobiopterin	0.003 (6.0)
L-*erythro*-Biopterin	0 (4.5～8.0)
Sepiapterin	0 (4.5～8.0)
6-Methylpterin	0.040 (6.0)
6-Hydroxymethylpterin	0.041 (6.0)
6-Carboxypterin	0.002 (6.0)
6-Formylpterin	0.001 (6.0)
Pterin	0.010 (6.0)
Xanthopterin	0.017 (7.0)[c]
Isoxanthopterin	0 (4.5～8.0)
Leucopterin	0 (4.5～8.0)

[a] Enzyme activity is defined as the change in absorbance at 340 nm observed for one min.
[b] The number in parentheses indicates the pH of the assay.
[c] Enzyme activity was determined by measuring the decrease in absorbance at 390 nm under standard assay conditions in the absence of 30 mM 2-mercaptoethanol.

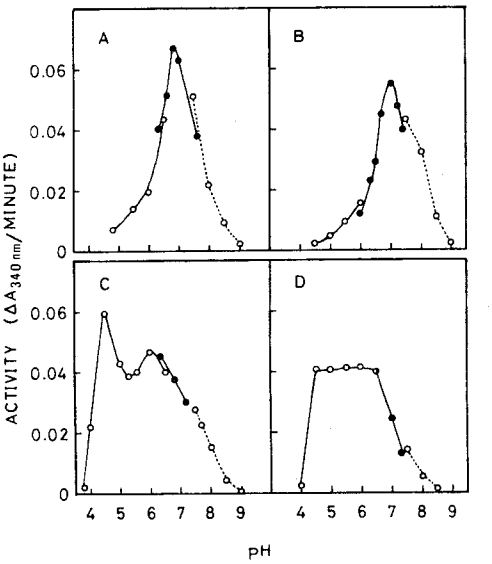


FIG. 2. Effect of pH on the Enzymic Reaction using Dihydrofolate (A), Dihydropteroate (B), L-*threo*-Neopterin (C) and 6-Hydroxymethylpterin (D) as Substrates.
○—○, sodium citrate buffer; ●—●, potassium phosphate buffer; ○---○, Tris-HCl buffer.

the enzyme had a broad specificity for pteridine compounds, as well as for dihydrofolate. Dihydropteroate, four neopterin isomers (L-*threo*-, L-*erythro*-, D-*threo*- and D-*erythro*-), 6-hydroxymethylpterin, 6-methylpterin and pterin worked as effective substrates. However, pteroate, 6-carboxypterin and 6-formylpterin were poor substrates. Xanthopterin in aqueous solution shows a spontaneous decrease in absorbance at 390 nm because of formation of 7,8-dihydro-7-hydroxy-xanthopterin.[16,17] However, the rapid decrease in absorbance at

390 nm was caused by addition of NADPH to the complete reaction mixture at pH 7.0. Folate, sepiapterin, L-*erythro*-biopterin, isoxanthopterin and leucopterin were not reduced at any pH range between 8.0 and 4.5. The reduction of L-*erythro*-7,8-dihydrobiopterin was 20% of that of L-*threo*-7,8-dihydroneopterin.

Each of the reactions having a pteridine compound as the substrate required NADPH as the cofactor, and NADH could not replace the NADPH.

The pH-activity profiles for dihydrofolate, dihydropteroate, 6-hydroxymethylpterin and L-*threo*-neopterin are illustrated in Fig. 2. For the former two compounds, the pH optima were 6.8 and 7.0, respectively. A broad optimum between pH 6.5 and 4.5 was shown for 6-hydroxymethylpterin, while L-*threo*-neopterin had a double optimum at pH 6.0 and 4.5.

Kinetic parameters The apparent *Km* values for dihydrofolate, dihydropteroate, 6-hydroxymethylpterin and L-*threo*-neopterin and for NADPH are given in Table II. The *Km* values of the enzyme for dihydrofolate and NADPH were higher than those of fraction IIa (1.1 and 2.7 μM, respectively).[1] The *Km* values for the two unconjugated pteridines were similar to that for dihydrofolate.

TABLE II. *Km* VALUES FOR DIHYDROFOLATE, DIHYDROPTEROATE, 6-HYDROXYMETHYLPTERIN, L-*threo*-NEOPTERIN AND NADPH

Enzyme activity was assayed under standard assay conditions. The apparent *Km* values were calculated from Lineweaver-Burk plots.

Compound	pH of assay	*Km* for compound (μM)	*Km* for NADPH[a] (μM)
Dihydrofolate	6.8	4.8	5.9
Dihydropteroate	7.0	0.9	2.1
6-Hydroxymethylpterin	6.0	3.4	5.9
L-*threo*-Neopterin	6.0	3.5	11

[a] These *Km* values were calculated from data collected in the presence of both the compound and NADPH.

TABLE III. *Ki* VALUES FOR INHIBITION OF THE PTERIDINE REDUCTASE : DIHYDROFOLATE REDUCTASE ACTIVITY BY BIOPTERIN, FOLATE, METHOTREXATE, PYRIMETHAMINE, TRIMETHOPRIM AND NADP

Each substrate was added after preincubation of the enzyme with the inhibitor for 3 min at 30 C. The reaction was initiated by the addition of NADPH. Both dihydrofolate reductase and pteridine reductase activities were measured under standard assay conditions. The *Ki* values were calculated from Dixon plots.[19]

Inhibitor	*Ki* (μM)		
	Substrate		
	Dihydrofolate (6.8)[a]	L-*threo*-Neopterin (6.0)[a]	6-Hydroxymethylpterin (6.0)[a]
L-*erythro*-Biopterin	0.34	0.040	0.061
Folate	36	27	40
Methotrexate	1.1	0.096	0.33
Pyrimethamine	0.72	0.24	0.48
Trimethoprim	6.4	2.6	4.0
NADP	21	10	11

[a] pH of the assay.

but, the *Km* value for NADPH in the presence of L-*threo*-neopterin was higher than those of the other three substrates.

As shown in Table III, both PtR and DFR activities were inhibited by folate, L-*erythro*-biopterin, NADP and anti-folates, such as MTX, pyrimethamine and trimethoprim. The magnitude of inhibition by both biopterin and MTX depended upon the pteridine compound used as the substrate and not on the dihydrofolate. Thus, the PtR activity was inhibited to a greater extent by biopterin and MTX than was the DFR activity. Both activities were also inhibited by folate, NADP, pyrimethamine and trimethoprim at similar concentrations. The presence of folate, biopterin, MTX, pyrimethamine or trimethoprim produced competitive inhibition with the pteridine compounds and dihydrofolate (as estimated from Dixon plots[18]). The inhibition by NADP was also competitive with NADPH.

Reaction product Changes in the absorption spectrum of the

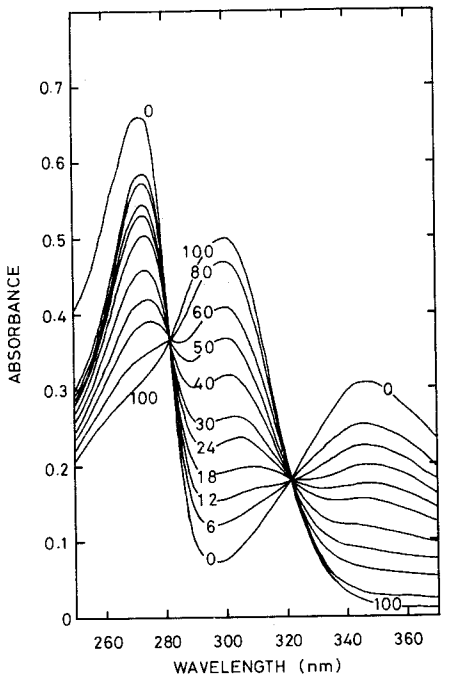


FIG. 3. Changes in the Absorption Spectrum of 6-Hydroxymethylpterin during Its Reduction by Pteridine Reductase : Dihydrofolate Reductase.

The reaction mixture contained the following: 50 mM potassium phosphate, pH 7.0; 30 mM 2-mercaptoethanol; 50 μM 6-hydroxymethylpterin; 20 μM NADPH; 10 mM glucose-6-phosphate; glucose-6-phosphate dehydrogenase (2.8 units); pteridine reductase : dihydrofolate reductase (360 μg protein). Final volume was 2.5 ml. The spectrum changes were recorded at 6 min intervals for a period of 100 min.

pteridine compound during the enzymic reaction were measured by coupling the reaction to the NADPH-regenerating system of glucose-6-phosphate dehydrogenase. As illustrated in Fig. 3, the spectrum of 6-hydroxymethylpterin(λmax 273 and 346 nm at pH 7.0) changed to one having a λmax at 300 nm. The isosbestic points of the spectrum during this reaction were 321 and 281 nm, and the spectrum of the final product was identical to that of 6-hydroxymethyltetrahydropterin. When other pteridine compounds were used as substrates, similar spectral changes were observed. The spectral changes of the conversion of dihydrofolate to tetrahydrofolate were in good agreement with the data reported by Osborn and Huennekens.[14]

NADPH-consumption during the enzymic reaction The amount of NADPH consumed during the enzymic reduction of L-*threo*-neopterin was determined by measuring the decrease in absorbance at 340 nm, as shown in Fig. 4. There was a rapid decrease for the first 12

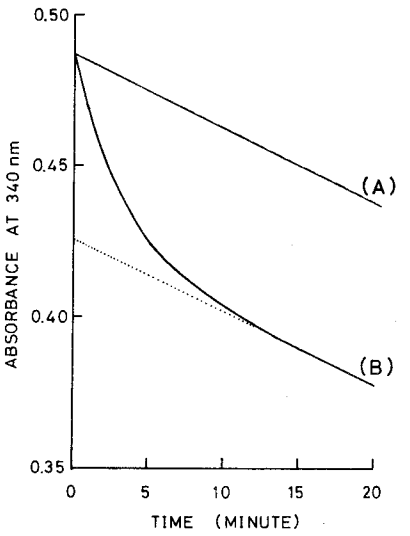


FIG. 4. NADPH-Consumption during the Enzymic Reduction of L-*threo*-Neopterin.

The reaction mixture contained 3.6 μM L-*threo*-neopterin, 30 mM 2-mercaptoethanol and pteridine reductase : dihydrofolate reductase (190 μg protein) in 50 mM sodium citrate buffer at pH 6.0. The reaction was initiated by adding 76 μM NADPH to a mixture without enzyme (A), or to that containing enzyme (B).

min, and then the absorbance continued to decrease a slower, but linear, fashion. Since this linear decrease was also observed in a mixture containing neither the enzyme nor neopterin, the auther concluded, along with Hillcoat *et al.*, [19] that this decrease was due to decomposition of the NADPH at pH 6.0. Thus, the absorbancy changes between line (A) and an extrapolated line (B) were consequences of the conversion of both NADPH to NADP and L-*threo*-neopterin to L-*threo*-tetrahydroneopterin. The amount of oxidized NADPH was calculated to be 6.9 μM, based on the molar extinction coefficient for reduction of 3.6 μM of L-*threo*-neopterin. Therefore, it was estimated that two mol of NADPH were used for the reduction of one mol of L-*threo*-neopterin. When dihydrofolate was used as a substrate, 1.1 mol of NADPH were used for reduction of one mol of dihydrofolate.

Effect of sulfhydryl reagents and chaotropes As shown in Table IV, the DFR activity was inhibited by sulfhydryl reagents, such as pCMB and NEM, and by chaotropes, such as guanidine-HCl, formamide and urea. The relative inhibition of DFR activity pro-

TABLE IV. EFFECT OF SULFHYDRYL REAGENTS AND CHAOTROPES ON DIHYDROFOLATE REDUCTASE ACTIVITY

After the enzyme was preincubated with the reagent for 5 min at 30°C, the dihydrofolate reductase activity was measured under standard assay conditions as described previously.[1] The activity of the treated enzyme is expressed relative to that of the untreated enzyme.

Reagent		Relative activity (%)
None		100
pCMB[a]	1.2 μM	42.3
	1.2 μM + 8 mM 2-ME[b]	62.4
NEM[c]	0.2 mM	33.8
	0.2 mM + 8 mM 2-ME[b]	56.2
Guanidine-HCl	0.08 M	97.2
	0.2 M	66.5
Formamide	0.8 M	91.4
	1.6 M	72.3
Urea	2.0 M	46.7
	4.0 M	6.7

[a] *p*-Chloromercuribenzoate.
[b] 2-Mercaptoethanol.
[c] *N*-Ethylmaleimide.

duced by each reagent was similar to that obtained from fraction IIa.[1] (see Chapter II)

DISCUSSION

In this Chapter, the fraction IIb from *C. fasciculata*, having both DFR and PtR activities, was further purified and its properties were characterized. The purified fraction IIb resembled fraction IIa, *i.e.*, dihydrofolate and NADPH-dependent DFR,[1] in its pH-activity curve using dihydrofolate as a substrate (Fig. 2,A), its molecular weight (110,000) and its sensitivities to trimethoprim, pyrimethamine, pCMB, NEM, guanidine-HCl, formamide and urea (Table III and IV). However, the purified fraction IIb differed from the fraction IIa in the following physical properties:

(1) The IIb protein was not adsorbed on CM-Sephadex in the

presence of 2-ME, and was stable in the presence of ammonium sulfate and 2-ME.

(2) Fraction IIb exhibited both DFR and PtR activities. That is, it catalyzed the reduction of many pteridine compounds, such as 6-hydroxymethylpterin, 6-methylpterin, four neopterin isomers, pterin and xanthopterin, to their tetrahydro forms, as well as the reduction of dihydrofolate and dihydropteroate in the presence of NADPH (Table I and Fig. 3).

(3) MTX is a potent inhibitor of many DFRs,[1,3,4] including fraction IIa (see Chapter II). Both the DFR and PtR activities of the IIb protein were inhibited competitively by MTX (Table III). However, the concentration required for 50% inhibition of the DFR activity was 5,500 times higher than that required to inhibit the fraction IIa protein (7.7 μM *versus* 1.4 nM for IIa).

(4) Both the DFR and the PtR activities of fraction IIb were also strongly inhibited by L-*erythro*-biopterin (Table III), and the *Ki* value for the IIb DFR activity was 382 times lower than that for fraction IIa (0.34 μM *versus* 130 μM for IIa). L-*erythro*-Biopterin has not yet been reported as an inhibitor of any DFR.

Some DFRs reduce folate,[2-4] pteroate,[11,20-22] dihydropteroate,[10,11,20,21,23,24] dihydrobiopterin[12,25] and 6-methyldihydropterin.[11,23,25] The enzyme from chicken liver reduces biopterin at a very low rate,[12] while folate reductase from chicken liver reduces the aldehyde of 6-formylpterin.[22] Up to this time, a DFR or other reductase which can effectively reduce oxidized pteri-

dine compounds to tetrahydroforms has been unknown. However, the data obtained in these experiments suggest that the enzyme from fraction IIb is quite a different type from any DFR isolated thus far. Therefore, it was named pteridine reductase:dihydrofolate reductase. Additionally, it was concluded that the enzyme activity depends on the structure of the pteridine side chain at the 6-position, as well as its stereo configuration, since the enzyme is unable to use folate, L-*erythro*-biopterin, sepiapterin, isoxanthopterin and leucopterin as substrates.

Recently, Hirayama *et al.*[26] have isolated dihydropteridine reductase from *C. fasciculata* ATCC 11745, which reduces quinonoid-6-methyldihydropterin to 6-methyltetrahydropterin in the presence of NADH as a cofactor. However, this enzyme differs from PtR:DFR in such properties as substrate specificity and molecular weight (55,000 daltons).

REFERENCES

1) K. Iwai, M. Kohashi and H. Oe, *Agric. Biol. Chem.*, 45, 113 (1981).

2) F. M. Huennekens, R. B. Dunlap, J. H. Freisheim, L. E. Gundersen, N. G. L. Harding, S. A. Levison and G. P. Mell, *Ann. N. Y. Acad. Sci.*, 186, 85 (1971).

3) F. M. Huennekens, "Biological Oxidation," ed. by T. P. Singer, Interscience, New York, 1968, p. 439.

4) R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines," North-Holland Publ. Co., Amsterdam, London, 1969,

p. 139.

5) R. Ferone, J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, 5, 49 (1969).

6) E. G. Platzer, *J. Protozool.*, 21, 400 (1974).

7) W. E. Gutteridge, J. J. Jaffe and J. J. McCormack, Jr., *Biochim. Biophys. Acta*, 191, 753 (1969).

8) W. E. Gutteridge, J. J. McCormack, Jr. and J. J. Jaffe, *Biochim. Biophys. Acta*, 178, 453 (1969).

9) R. Ferone and S. Roland, *Proc. Natl. Acad. Sci. U. S. A.*, 77, 5802 (1980).

10) R. Nath and D. M. Greenberg, *Biochemistry*, 1, 435 (1962).

11) D. R. Morales and D. M. Greenberg, *Biochim. Biophys. Acta*, 85, 360 (1964).

12) S. Kaufman, *J. Biol. Chem.*, 242, 3934 (1967).

13) M. Matsubara, S. Katoh, M. Akino and S. Kaufman, *Biochim. Biophys. Acta*, 122, 202 (1966).

14) M. J. Osborn and F. M. Huennekens, *J. Biol. Chem.*, 233, 969 (1958).

15) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193, 265 (1951).

16) Y. Inoue and D. D. Perrin, *J. Chem. Soc.*, 2600 (1962).

17) M. A. Schou, *Arch. Biochem.*, 28, 10 (1950).

18) M. Dixon, *Biochem. J.*, 55, 170 (1953).

19) B. L. Hillcoat, P. F. Nixon and R. L. Blakley, *Anal. Biochem.*, 21, 178 (1967).

20) P. F. Nixon and R. L. Blakley, *J. Biol. Chem.*, 243, 4722 (1968).

21) J. R. Bertino, J. P. Perkins and D. G. Johns, *Biochemistry*, 4, 839 (1965).

22) S. F. Zakrzewski, *J. Biol. Chem.*, 235, 1780 (1960).

23) D. M. Greenberg, B.-D. Tam, E. Jenny and B. Payes, *Biochim. Biophys. Acta*, 122, 423 (1966).

24) L. T. Plante, E. J. Crawford and M. Friedkin, *J. Biol. Chem.*, 242, 1466 (1967).

25) S. Matsuura, T. Sugimoto, H. Hasegawa, S. Imaizumi and A. Ichiyama, *J. Biochem.*, 87, 951 (1980).

26) K. Hirayama, N. Nakanishi, T. Sueoka, S. Katoh and S. Yamada, *Biochim. Biophys. Acta*, 612, 337 (1980).

c) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 251 (1983).

Chapter IV

Effect of Configuration of Neopterin Isomers on the Activity of Pteridine Reductase:Dihydrofolate Reductase from *Crithidia fasciculata* [d)]

As described in Chapter III,[1)] PtR:DFR from *C. fasciculata* reduces one mol each of oxidized pteridine compounds such as four neopterin isomers (*i.e.*, L-*threo*, L-*erythro*, D-*threo* and D-*erythro*-neopterins), 6-hydroxymethylpterin, 6-methylpterin and pterin to their tetrahydro forms using 2 mol of NADPH. The reductase activity for neopterin isomers at pH 6.0, however, varies with their structures.[1)] The enzyme is unable to reduce L-*erythro*-biopterin and sepiapterin at any pH range tested.[1)] This suggests that the CH_2OH-group at C-3' of the propyl side chain of pteridine is an essential structure for the substrate of PtR:DFR and also the configuration of the trihydroxypropyl side chain of neopterin affects the activity of PtR:DFR.

The present chapter describes the PtR:DFR activity in relation to the structure of four stereoisomers of neopterin in order to clarify the reduction mechanism of PtR:DFR. The data show that the activity of PtR:DFR depended much on the configuration of OH at C-1' of the trihydroxypropyl side chain of neopterin as a substrate.

MATERIALS AND METHODS

Materials. NADPH was obtained from Sigma Chemical Co. L-*threo*-Neopterin was a kind gift from Dr. M. Viscontini, Zürich University, Switzerland. The other three isomers of neopterin were synthesized by the method described by Viscontini and Provenzale.[2)]

Standard assay conditions for PtR:DFR. The PtR:DFR activity was measured by the method described in Chapter III.[1)] The enzyme activity was defined as the decrease in absorbance at 340 nm for one min under the standard assay conditions.[1)]

RESULTS AND DISCUSSION

Optimum pH and kinetic parameters The pH-activity profiles for the four stereoisomers of neopterin, *i.e.*, L-*threo*, L-*erythro*, D-*threo* and D-*erythro*-neopterin, are shown in Fig. 1. The Michaelis constants (*Km* value) for NADPH and neopterin isomers at the optimum pHs are summarized in Table I. L-*threo*-Neopterin showed two pH optima at pH 6.0 and 4.5, as described in the preceding Chapter III.[1)] The *Km* value for L-*threo*-neopterin at pH 4.5 was 1.9 times higher than that at pH 6.0. The enzyme activity at pH 4.5 was inhibited by over 20 μM L-*threo*-

TABLE I. *Km* VALUES FOR NADPH AND STEREOISOMERS OF NEOPTERIN FOR PTERIDINE REDUCTASE : DIHYDROFOLATE REDUCTASE ACTIVITY

The enzyme activity was assayed at 340 nm under the standard conditions as described in the previous paper.[1)] The enzyme (90 μg of protein) was preincubated at 30°C for 3 min. The reaction was initiated by adding 60 ~ 80 μM neopterin and 80 μM NADPH in that order.

Neopterin	Optimum pH	*Km* for neopterin (μM)	*Km* for NADPH* (μM)
L-*threo*-Neopterin	6.0	3.5	11
	4.5	6.7	4.7
L-*erythro*-Neopterin	6.0	6.4	21
D-*threo*-Neopterin	4.8	37	13
D-*erythro*-Neopterin	4.5	11	23

* *Km* values in the presence of each neopterin.

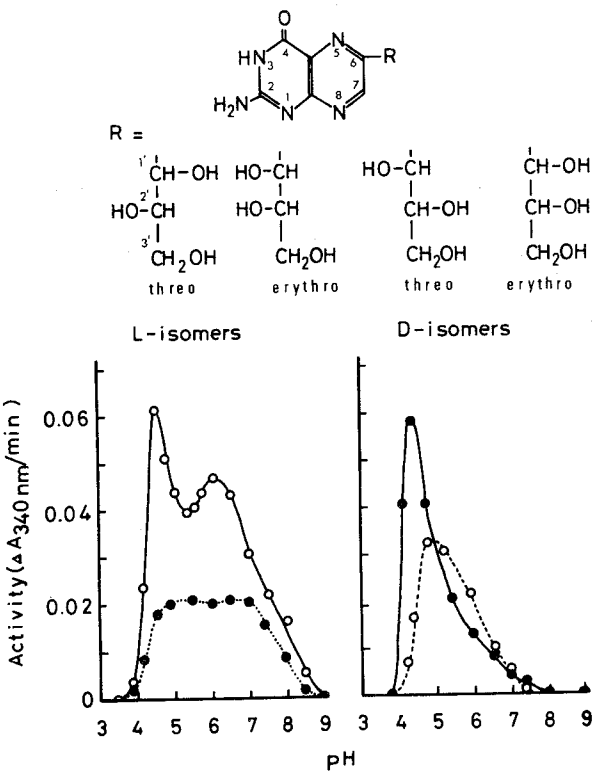


FIG. 1. Effect of pH on the Activity of Pteridine Reductase: Dihydrofolate Reductase with Four Stereoisomers of Neopterin.
(○—○), L-*threo*-neopterin; (●---●), L-*erythro*-neopterin; (○---○), D-*threo*-neopterin; (●—●), D-*erythro*-neopterin.

neopterin. The constant (*Ks'* value) for the substrate inhibition was 95 μM. However, the *Km* value for NADPH in the presence of L-*threo*-neopterin at pH 4.5 was 2.3 times lower than that at pH 6.0. A broad optimum pH between 7.0 and 4.5 was shown for L-*erythro*-neopterin (Fig. 1). This pH-activity profile was similar to that of 6-hydroxymethylpterin,[1)] although its maximum activity was half that of 6-hydroxymethylpterin. The *Km* value for L-*erythro*-neopterin at pH 6.0 was 1.8 times higher than that of L-*threo*-neopterin at the same pH, and the *Km* value for NADPH in the presence of the L-*erythro*-isomer was 1.9 times higher than that of NADPH in the

presence of the L-*threo*-isomer. On the contrary, a single pH optimum was shown at 4.5 and 4.8 for D-*erythro* and D-*threo*-neopterins, respectively (Fig. 1). The maximum activity for D-*erythro*-neopterin was almost two times higher than that for D-*threo*-neopterin. The *Km* value for D-*erythro*-neopterin was 3.4 times lower than that for D-*threo*-neopterin.

As shown in Fig. 1, the configuration of the hydroxy group at C-1' of L-*threo*-neopterin was the same as that of D-*erythro*-neopterin, and the configuration of L-*erythro*-isomer was the same as that of D-*threo*-isomer. As shown in Table I, PtR:DFR reduced more favorably L-*threo* and D-*erythro*-neopterins than L-*erythro* and D-*threo*-neopterins. As shown in Chapter III, PtR:DFR reduces L-*erythro*-7,8-dihydrobiopterin, but not L-*erythro*-biopterin.[1] This suggests that the activity of PtR:DFR is controlled by the configuration at C-1' during the reduction at C-7 and N-8 of the pteridine ring. Charlton *et al.*[3] have reported that the hydrogen at the 4-*pro*-R position of NADPH is transferred to the *si*-face at C-7 of folate during folate reduction by DFR. When the hydrogen transfer from NADPH takes place in a similar manner to the enzymic reduction of folate during the reduction of neopterins, the hydroxy group at C-1' of L-*erythro* and D-*threo*-neopterins may exist on the *si*-face at C-7 of the pteridine ring and cause steric hindrance of the reduction at C-7. Therefore, the hydroxy group of L-*threo* and D-*erythro*-neopterins may exist on the *re*-face at C-7, this configuration facilitating the hydrogen transfer from NADPH to neopterins.

This indicates that L-*threo* and D-*erythro*-neopterins are more favorable substrates for PtR:DFR than D-*threo* and L-*erythro*-isomers, which corresponds well with the data shown in Table I.

The estimation of the absolute configuration at C-6 of 5,6,7,8-tetrahydroneopterins may also be important for clarifying the reaction mechanism. The configuration at C-6 of tetrahydrofolate produced from 7,8-dihydrofolate[4,5] or folate[3] by DFR is estimated to be the *S*-form. The configuration at C-6 of 6-methyltetrahydropterin[4,6,7] is the *S*-form, and that of L-*erythro*-tetrahydrobiopterin[6,8-10] is the *R*-form. This suggests that the absolute configuration at C-6 of tetrahydroneopterins produced by PtR:DFR is the *R*-form.

REFERENCES

1) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 251 (1983).

2) M. Viscontini and R. Provenzale, *Helv. Chim. Acta*, 51, 1495 (1968).

3) P. A. Charlton, D. W. Young, B. Birdsall, J. Feeney and G. C. K. Roberts, *J. Chem. Soc. Chem. Commun.*, 922 (1979).

4) W. L. F. Armarego, P. Waring and J. W. Williams, *J. Chem. Soc. Chem. Commun.*, 334 (1980).

5) W. L. F. Armarego and P. Waring, *J. Chem. Research (S).*, 318 (1980).

6) S. Matsuura, T, Sugimoto, H. Hasegawa, S. Imaizumi and A. Ichiyama, *J. Biochem.*, 87, 951 (1980).

7) S. Matsuura and T. Sugimoto, *Bull. Chem. Soc. Jpn.*, 54, 2543 (1981).

8) S. W. Bailey and J. E. Ayling, *J. Biol. Chem.*, 253, 1598 (1978).

9) H. Hasegawa, S. Imaizumi, A. Ichiyama, T. Sugimoto, S. Matsuura, K. Oka, T. Kato, T. Nagatsu and M. Akino, "Chemistry and Biology of Pteridines" ed. by R. L. Kisliuk and G. M. Brown, Elsevier, North-Holland, New York, 1979, p. 183.

10) T. Kato, T. Yamaguchi, T. Nagatsu, T. Sugimoto and S. Matsuura, *Biochim. Biophys. Acta*, 611, 241 (1980).

d) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 425(1983).

Chapter V

Uptake and Metabolism of Methotrexate by *Crithidia fasciculata*[e)]

Crithidia fasciculata has an energy-dependent active transport system for folate.[1)] In Chapter I, MTX and aminopterin promote the growth rate of *C. fasciculata* at the same concentrations as does folate.[2)] In Chapters II and III, MTX inhibits the DFR activity,[3,4)] but there is no difference in the types and amounts of this enzyme between cells grown in media containing either MTX or biopterin.[2)] This suggests that *C. fasciculata* may metabolize MTX to compounds which can be used effectively for its own growth and have no inhibitory effect on the DFR activity.

Transport of MTX in L1210 lymphoma cells[5-7)] and *Lactobacillus casei*[7)] is an active, energy-dependent, carrier-mediated process. The incorporated MTX is metabolized mainly to 7-hydroxy MTX[8-14)] or its poly-γ-glutamyl compounds[14-22)] by mammals, and to AMPte by bacteria.[22-26)] This chapter deals with the uptake of MTX and the isolation of a major metabolite of MTX in *C. fasciculata*. The significance of the occurrence of an enzyme activity converting MTX to AMPte in this protozoan is discussed.

MATERIALS AND METHODS

Materials. [3',5',9(n)-^{3}H]MTX sodium salt (6 Ci/mmol) and [2-^{14}C]folic acid potassium salt (54.3 mCi/mmol) were purchased from

the Radiochemical Centre (Amersham). MTX was purchased from Lederle Ltd. POPOP and PPO were from Wako Pure Chemicals Industries Ltd. Avicel SF cellulose plates (20 x 20 cm) were from Funakoshi Pharmaceuticals Co. Ltd. Membrane filters (0.45 μm in pore diameter) were from Millipore Co. DEAE-cellulose and Sephadex G-10 were from Pharmacia Fine Chemicals. All other chemicals were obtained from Nakarai Chemicals Ltd., Kyoto.

Media and growth conditions for C. fasciculata. *C. fasciculata* was grown at 25°C in the chemically defined assay medium described by Guttman.[27] MTX (0.1 μM) or L-*erythro*-biopterin (0.21 nM) was aseptically added to the sterilized medium (10 ml) by filtering through a Millipore filter. The cell growth was measured turbidimetrically with a Coleman Model 6-20 Spectrophotometer and shown by transmittance (T%) at 675 nm. The cell number was determined microscopically using a haematometer (depth 0.1 mm).

Uptake of MTX and folate. The uptake studies of MTX and folate were conducted using cells grown in the chemically defined medium (10 ml) containing 0.21 nM L-*erythro*-biopterin. After a defined interval of growth, 5.64 nM [^{3}H]MTX (333 nCi) or 115 nM [^{14}C]folate (61.8 nCi) was added to the culture medium and incubated at 25°C for 1 to 20 min. The cells in 2 ml of the medium were collected with a Millipore filter and washed thoroughly with an ice-cold solution containing 0.5% (w/v) sodium chloride, 0.5% (w/v) potassium chloride and 0.1 mg% (w/v) MTX or folate. The filter was put in a counting vial containing 10 ml of scintillation fluid

consisting of 0.1 g of POPOP and 5.5 g of PPO dissolved in one liter of a mixture of toluene and Triton-X 100 (2:1, v/v). The radioactivity was measured by a Packard 2425 Tri-Carb Liquid Scintillation Spectrometer.

In order to quantify the MTX taken up by the cells, cells grown in the chemically defined medium containing 0.1 μM [^{3}H]MTX (516 nCi) were collected at a defined interval of growth from 2 ml of the medium, and the radioactivity recovered in the cells was measured as described above.

Isolation of metabolites of MTX from the cells and the medium.

The cells were cultivated in 100 ml of the chemically defined medium containing 0.1 μM [^{3}H]MTX (130 nCi) until their concentration reached T=40 - 50%, and harvested by centrifugation at 3,000 x *g* for 10 min. The resulting cells (0.2 g) were washed 3 times with the ice-cold solution as described above (see *Uptake of MTX and folate*) and suspended in 3 ml of 10 mM Tris-HCl buffer, pH 7.0, containing 100 mM 2-ME. The cells were disrupted for 2 min on ice with a Kaijo-Denki 20 kHz Ultrasonic Oscillator, and the suspension was centrifuged at 12,000 x *g* for 30 min. The resulting supernatant solution was put on a DEAE-cellulose column (1.4 x 7 cm) which had been equilibrated with the Tris-HCl buffer. Also, the culture supernatant (10 ml) was diluted to 20 ml with 200 mM 2-ME, the pH was adjusted to 7.0 with 1 M NaOH, and the solution was put on another DEAE-cellulose column as described above. The columns were eluted by a linear Tris gradient with 60 ml of 10 mM Tris-HCl buffer,

pH 7.0, containing 100 mM 2-ME in the mixing chamber and 60 ml of 1 M Tris-HCl buffer, pH 7.0, containing 100 mM 2-ME in the reservoir. Two milliliter fractions were collected, and an aliquot (0.2 ml) of the fraction was used for radioassay. About 95% of the total radioactivity was recovered from the column. Each radioactive fraction (I - V) was pooled and lyophilized. After being dissolved in a small amount of water, the radioactive solution was desalted by chromatography on a Sephadex G-10 column (1.5 x 85 cm) using water. The radioactive fractions were collected and lyophilized. All procedures used in the present experiments were carried out at 0 - 4°C.

Preparation of the cell-free extract. The cells were cultivated in 2 liters of the culture medium described by Guttman[27] until their concentration reached T=60%, harvested by centrifugation, and washed thoroughly with 0.9% (w/v) saline. The resulting cells (2 g) were suspended in 5 ml of 0.1 M Tris-HCl buffer, pH 7.1, and disrupted on ice by sonicating for 5 min. The suspension was centrifuged at 12,000 x *g* for 30 min. The supernatant solution was used for the following experiments.

Isolation of the enzymatic reaction product of MTX. The reaction mixture containing 0.1 M Tris-HCl buffer, pH 7.1, 16.7 μM [^{3}H] MTX (375 nCi) and the cell-free extract (4.4 mg of protein) in a total volume of 0.18 ml, was incubated at 30°C for 1 hr. The reaction was terminated by adding 0.18 ml of ethanol to the mixture. An aliquot of the supernatant obtained by centrifugation at 10,000 x *g* for 10 min was spotted on an Avicel SF cellulose plate. The

plate was developed with 3% (w/v) ammonium chloride solution at room temperature. The plate was scraped off in 1-cm segments and each segment was put in a counting vial containing 10 ml of a scintillation fluid consisting of 0.1 g of POPOP and 4 g of PPO dissolved in one liter of toluene.

To isolate a non-radioactive reaction product, MTX (0.1 mM) was incubated at 37°C for 2 hr in a mixture (1 ml) of 0.1 M Tris-HCl buffer, pH 7.1, and the cell-free extract (29 mg of protein). The reaction was terminated by the addition of ethanol. The insoluble materials were removed by centrifugation. The resulting supernatant solution was concentrated to a small volume and all the solutions were spotted on an Avicel SF cellulose plate. The plate was developed with 0.1 M potassium phosphate, pH 7.0, at room temperature. The purple zone (*Rf*=0.29 - 0.32) located under a Super-Light Model LS-D1, Nikko Sekiei Works (wavelength, 365 nm) was scraped off and eluted with 10 ml of water. After concentrating the eluate in an evaporator *in vacuo*, the yellow solution was desalted with a Sephadex G-10 column (1.5 x 85 cm) using water. The yellow fractions showing an absorbance at 280 nm were collected and lyophilized.

RESULTS

Cellular uptake of MTX and folate The uptake of MTX and folate by *C. fasciculata* is shown in Table I. It was found that the cellular capacity of MTX uptake (expressed per unit of cell

TABLE I. UPTAKE OF [^{3}H]METHOTREXATE AND [^{14}C]FOLATE BY *Crithidia fasciculata* CELLS OBTAINED FROM VARIOUS CULTIVATION TIMES

C. fasciculata was grown at 25°C in a chemically defined medium (10 ml) containing 0.21 nM L-*erythro*-biopterin. After growth intervals of 95, 105, 115, 120, 123, 125, 130 and 135 hr, [^{3}H]methotrexate (5.67 nM, 333 nCi) or [^{14}C]folate (115 nM, 61.8 nCi) was added to the culture medium, and the mixture was incubated at 25°C for 1 min. At the end of this incubation, the cells in a 2 ml-aliquot of the medium were collected with a Millipore filter, and the radioactivity bound to the cells was measured.

Cultivation time (hr)	Growth (T% at 675 nm)	Count of cells/10 ml ($\times 10^8$)	The amount taken up (fmol/10^8 cells)	
			Methotrexate	Folate
95	89	0.60	22	31
105	83	0.93	37	39
115	71	1.77	23	247
120	63	2.50	21	230
123	56	3.45	20	225
125	49	4.50	23	167
130	38	6.15	21	131
135	25	8.00	19	70

number) did not depend on the MTX concentration nor on cultivation time. The cells completed the MTX uptake within 1 min after the start of incubation with MTX, and no further uptake occurred throughout the following incubation period of 20 min. The amount of MTX taken up in 1 min was at a low level such as 19 to 37 fmol/10^8 cells. On the contrary, the cells obtained at each growth stage exhibited a linear fashion of folate uptake lasting at least for 20 min. The amounts of folate taken up by the cells varied from 31 to 247 fmol/10^8 cells during growth. The uptake rate of folate depended on the folate concentration in the medium. The apparent *Km* value (uptake constant) for folate was calculated to be 44 nM from the reciprocal plots of uptake rate *versus* folate concentration. The folate uptake was inhibited 28, 88 and 92% by 0.066, 6.6 and 120 μM of MTX, respectively, and also 92% by 120 μM of aminopterin.

Amounts of MTX taken up by the cells during growth When the

Crithidia cells were cultivated in a medium containing MTX, the radioactivity derived from MTX which was taken up by the cells increased during growth (Fig. 1).

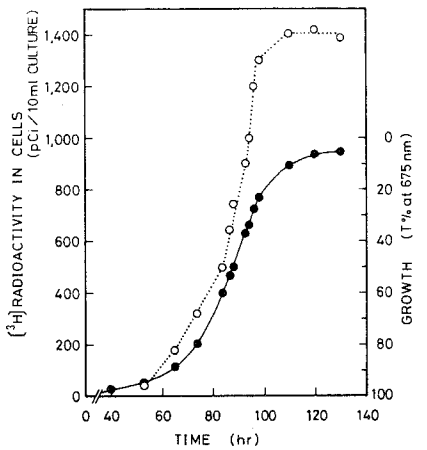


FIG. 1. Amount of Methotrexate Taken Up by *Crithidia fasciculata* Cells during Growth.

C. fasciculata was grown in a chemically defined medium (10 ml) containing 0.1 μM [^{3}H]methotrexate (516 nCi). ●——●, cell growth; —o--o—, total radioactivity in cells.

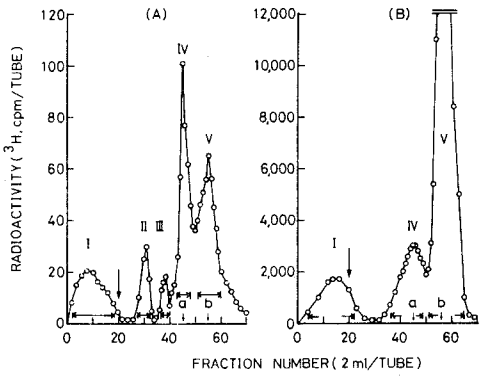


FIG. 2. DEAE-Cellulose Chromatography of [^{3}H]Methotrexate-metabolites Found in the Cells of *C. fasciculata* (A) and the Culture Supernatant (B).

C. fasciculata was grown in a chemically defined medium (100 ml) containing 0.1 μM [^{3}H]methotrexate (130 nCi). After 4 days of growth, the cells were separated from the medium as described in the text. The columns (1.4 × 7 cm) were eluted by a linear gradient of 0.01 ~ 1 M Tris-HCl buffer, pH 7.0. The gradients were started at the positions indicated by the arrows in the chromatograms. The symbols **a** and **b** in the chromatograms represent the eluting positions of authentic AMPte and methotrexate, respectively.

The amounts (as MTX) taken up by the cells at mid-log phase (T=50%) was calculated to be 330 fmol/10^8 cells from the radioactivity.

Metabolites of MTX

The elution profiles from a DEAE-cellulose column of the MTX metabolites found in the cell extract and the culture supernatant are shown in Figs. 2A and 2B, respectively. Five [^{3}H] radioactive fractions [I, II, III, IV and V(Fig. 2A)] and three fractions[I, IV and V(Fig. 2B)] were obtained from the cell extract and the culture supernatant, respectively. The eluting positions of the fractions IV and V corresponded to those of authentic AMPte and MTX, respectively. The

TABLE II. COCHROMATOGRAPHY OF FRACTION IV COMPOUND WITH AUTHENTIC AMPte

The fraction IV compounds (400 cpm and 510 cpm, respectively, see Figs. 2A and 2B) obtained by DEAE-cellulose chromatography of the cell extract and of the culture supernatant were cochromatographed with authentic AMPte on an Avicel SF cellulose plate. The zone corresponding to AMPte was scraped off and radioactivity was measured.

Solvent system[a]	Fraction IV compound from					
	Cell extract			Culture supernatant		
	Rf	Radioactivity (cpm)	Yield[b] (%)	*Rf*	Radioactivity (cpm)	Yield[b] (%)
a	0.31	363	91	0.29	465	91
b	—	—	—	0.25	494	97
c	0.32	380	95	0.31	493	97

[a] a, 0.1 M potassium phosphate buffer, pH 7.0; b, *n*-propanol–ethyl acetate–water, 7 : 1 : 2 (v/v/v); c, *n*-propanol–1% ammonium hydroxide, 2 : 1 (v/v).

[b] Recovery against radioactivity applied for the cellulose plate chromatography.

radioactive fraction IV was cochromatographed with authentic AMPte, and the major radioactivity was observed at the AMPte zone as shown in Table II. The radioactive fraction V also cochromatographed with MTX, and the radioactivity was observed at the MTX zone (data not shown). From these results, fraction IV and V were identified as AMPte and MTX, respectively. The radioactivities recovered as AMPte and MTX in the cell extract were found to be approximately 30 and 39% of the total activity, respectively. The fraction I which passed through the DEAE-cellulose column, and the minor fractions (II and III) were not identified.

Enzymatic conversion of MTX to AMPte by the cell-free extract

When [^{3}H]MTX was incubated with the cell-free extract, a new radioactive compound (*Rf* value of 0.27) was observed on thin-layer chromatography (Fig. 3). The infrared and UV spectra of this compound are shown in Figs. 4 and 5, respectively. The spectra

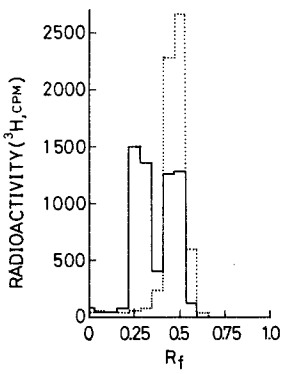


FIG. 3. Thin-layer Chromatograms of [^{3}H]Methotrexate before and after Incubated with the Cell-free Extract of *C. fasciculata*.

[^{3}H]Methotrexate (16.7 μM, 375 nCi) was incubated with the cell-free extract of *C. fasciculata* at 30°C for 1 hr. Twenty μl of the supernatant of the reaction mixture was spotted on an Avicel SF cellulose plate. See the text in details.

----, radioactivity profile on the chromatogram before the reaction; ——, that after the reaction.

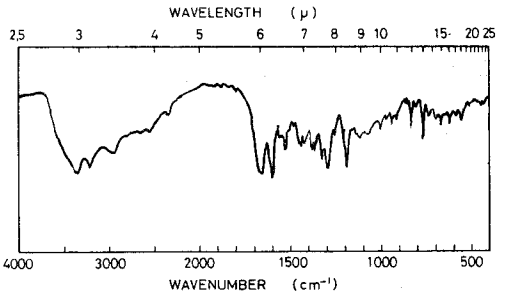


FIG. 4. Infrared Absorption Spectrum of the New Compound (see Fig. 3) Derived from Methotrexate.

The spectrum was taken in a KBr pellet by a Hitachi 285 Grating Infrared Spectrophotometer.

were in good agreement with those of 4-amino-4-deoxy-10-methylpteroic acid (AMPte) as reported by Levy and Goldman[23] (Fig. 6). The converting activity of MTX to AMPte was not observed in the culture supernatant.

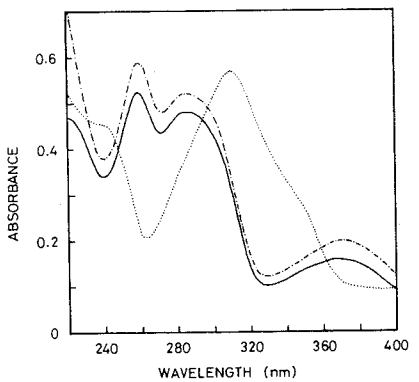


FIG. 5. Ultraviolet Absorption Spectra of the New Compound (see Fig. 3) Derived from Methotrexate in 0.1 M NaOH (-----), 0.1 M HCl (------) and 0.1 M Potassium Phosphate Buffer, pH 7.0 (——).

The spectra were taken by a Hitachi 124 Spectrophotometer.

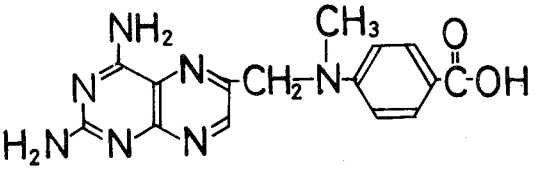


Fig. 6. Structure of 4-Amino-4-Deoxy-10-Methylpteroic Acid

DISCUSSION

The present study showed that the uptake of folate by *Crithidia fasciculata* depended on the degree of aging of the cells used (Table I) and on folate concentrations in the medium. However, the mode of the uptake of MTX differed from that of folate. Rembold and Vaubel[1] show that *C. fasciculata* has two different carrier sites for transporting biopterin and folate, based on their different responses to the addition of aminopterin. In the present study, MTX and aminopterin significantly inhibited the folate uptake, as observed by Rembold and Vaubel.[1] These results suggest that the uptake of MTX and aminopterin may share the carrier site for folate.

As shown in Fig. 2A, MTX taken up by *C. fasciculata* was metabolized to at least four compounds including AMPte. The intracellular amounts of the metabolized and unmetabolized MTX were calculated to be 200 and 130 fmol/10^8 cells, respectively, based on the results of Figs. 1 and 2A. *C. fasciculata* has two types of DFR, *i.e.*, DFR[3] and PtR:DFR[4] (see Chapters II and III). The concentration of the major DFR has been estimated to be 2.4 pmol per 10^8 cells at the same age.[2,3] Thus, the respective levels of the metabolites and MTX were 12- and 18-fold lower than that of the DFR. This estimation, about 5% of the DFR activity may be inhibited by the incorporated MTX, assumed that the dissociation of the enzyme-inhibitor complex is negligible. This is compatible with the results described in Chapter I.[2] The major metabolite, AMPte, has been shown as a weaker inhibitor for the DFR from *L. casei*,[14] mouse

L1210 leukemia cells[28] and P1534 leukemia cells[29] than MTX. Therefore, this evidence is interpreted to indicate that DFR of *C. fasciculata* may be little affected *in vivo* by concentrations of the MTX and AMPte.

The hydrolyzing reaction of MTX to AMPte has been reported in soil bacteria such as *Pseudomonas* sp.[23,24] and *Flavobacterium* sp.[25,26] The cell-free extract of *C. fasciculata* converted MTX to AMPte (Fig. 3) and folate to pteroic acid (data not shown). These results indicate that this protozoan may have an enzyme or enzymes catalyzing these hydrolytic conversions, as shown in soil bacteria.[23-26] Kidder *et al.*[30] has demonstrated that folate is metabolized *in vivo* to 6-hydroxymethylpterin and biopterin, which have a greater stimulatory effect on growth of *C. fasciculata*. But, much is not yet known about these metabolic pathway. A study is now in progress to elucidate which metabolites from MTX, including AMPte, have a growth-promoting activity in this protozoan. As the activity converting MTX to AMPte was only cell-bound, this conversion may play a role in the detoxication of MTX in this protozoan.

REFERENCES

1) H. Rembold and A. Vaubel, *Hoppe-Seyler's Z. Physiol. Chem.*, 351, 1277 (1970).

2) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 1651(1983).

3) K. Iwai, M. Kohashi and H. Oe, *Agric. Biol. Chem.*, 45, 113 (1981).

4) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 251 (1983).

:in and V. T. Oliverio, *J. Biol.*

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.iuk and G. M. Brown, Elsevier,

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lerer, Walter de Gruyter, Berlin,

1975, .

8) D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Booth and J. R. Bertino, *Biochim. Biophys. Acta.*, 105, 380 (1965).

9) H. M. Redetzki, J. E. Redetzki and A. L. Elias, *Biochem. Pharmacol.*, 15, 425 (1966).

10) E. Watson, J. L. Cohen and K. K. Chan, *Cancer Treat. Rep.*, 62, 381 (1978).

11) J. L. Wisnicki, W. P. Tong and D. B. Ludlum, *Cancer Treat. Rep.*, 62, 529 (1978).

12) S. A. Jacobs, R. G. Stoller, B. A. Chabner and D. G. Johns, *J. Clin. Invest.*, 57, 534 (1976).

13) W. P. Tong, J. L. Wisnicki, J. Horton and D. B. Ludlum, *Clin. Chim. Acta*, 107, 67 (1980).

14) S. K. Howell, Y.-M. Wang, R. Hosoya and W. W. Sutow, *Clin. Chem.*, 26, 734 (1980).

15) C. M. Baugh, C. L. Krumdieck and M. G. Nair, *Biochem. Biophys. Res. Commun.*, 52, 27 (1973).

16) D. S. Roseblatt, V. M. Whitehead, N. Vera, A. Pottier, M. Dupont

and M.-J. Vuchich, *Mol. Pharmacol.*, 14, 1143 (1978).

17) R. L. Schilsky, B. D. Bailey and B. A. Chabner, *Proc. Natl.Acad. Sci. U.S.A.*, 77, 2919 (1980).

18) U. Bühring, Y. S. Shin and E. Fölsch, *Cancer Res.*, 37, 299(1977).

19) D. A. Gewirtz, J. C. White, J. K. Randolph and I. D. Goldman, *Cancer Res.*, 39, 2914 (1979).

20) R. G. Poser, F. M. Sirotnak and P. L. Chello, *Cancer Res.*, 41, 4441 (1981)

21) M. Balinska, Z. Nimec and J. Galivan, *Arch. Biochem. Biophys.*, 216, 466 (1982).

22) Y. S. Shin, K. U. Buehring and E. L. R. Stokstad, *J. Biol.Chem.*, 249, 5772 (1974).

23) C. C. Levy and P. Goldman, *J. Biol. Chem.*, 242, 2933 (1967).

24) J. L. McCullough, B. A. Chabner and J. R. Bertino, *J. Biol.Chem.*, 246, 7207 (1971).

25) A. G. Pratt, E. J. Crawford and M. Friedkin, *J. Biol. Chem.*, 243, 6367 (1968).

26) A. M. Albrecht, E. Boldizsar and D. J. Hutchison, *J. Bacteriol.*, 134, 506 (1978).

27) H. N. Guttman,"Pteridine Chemistry," ed. by W. Pfleiderer and E. C. Taylor, Pergamon, London, 1964, p. 255.

28) D. Kessel, *Mol. Pharmacol.*, 5, 21 (1969).

29) D. M. Valerino, D. G. Johns, D. S. Zaharko and V. T. Oliverio, *Biochem. Pharmacol.*, 21, 821 (1972).

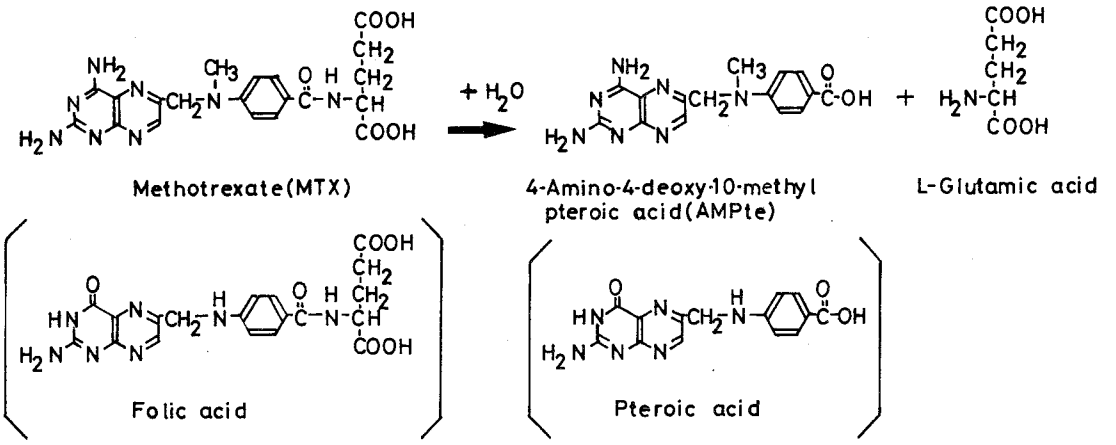
30) G. W. Kidder, V. C. Dewey and H. Rembold, *Arch. Mikrobiol.*, 59, 180 (1967).

e) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 1847 (1983).

Chapter VI

Radioassay of the Folate-hydrolyzing Enzyme Activity and the Distribution of the Enzyme in Biological Cells and Tissues[f)]

As shown in Scheme I, an enzyme which catalyzes the hydrolysis of MTX to AMPte and L-glutamic acid or of folic acid to pteroic acid and L-glutamic acid has been first isolated from a soil bacteria, *Pseudomonas* sp.[1)] and has been named as carboxypeptidase (CPase) G.[2)]



Scheme I

Various names of the enzyme which catalyzes the similar reaction have been proposed as follows: CPase G_1 from *Pseudomonas stutzeri*[3)] and folate amidase[4)] or CPase[5)] from *Flavobacterium* sp. The activities of these enzymes have been conveniently measured by a photometric method[1-5)] based on a change in absorbance of folic acid and MTX at 310 nm and 320 nm, respectively. In Chapter V, the auther has found that AMPte is accumulated in the cells of *C. fasciculata* grown

in the medium supplemented with MTX, and also has found that the cell-free extract of this protozoa has the hydrolyzing activity of MTX and folate to AMPte and pteroic acid, respectively.[6] However, these activities could not be detected by the photometric method. So, the auther developed a more sensitive radioassay method for analyzing the enzyme activity using [2-^{14}C]folic acid as a substrate, and analyzed the activity in microorganisms and mammalian tissues. In this chapter, the author named the hydrolyzing enzyme as the folate-hydrolyzing enzyme (FH-enzyme).

MATERIALS AND METHODS

Materials. The following chemicals were obtained from the specified manufacturers: [2-^{14}C]folic acid potassium salt (58.2 mCi/mmol) from The Radiochemical Centre (Amersham); PPO and POPOP from Packard Instrument Co.; pteroic acid from Lederle Ltd.; Avicel SF cellulose and its plates (20 x 20 cm) from Funakoshi Pharmaceutical Co.; Sephadex G-10 from Pharmacia Fine Chemicals; yeast extract from Oriental Yeast Co.; polypepton from Daigo-Eiyo Co.; beef extract from Mikuni Kagakusangyo Ltd.; thioglycollate medium from Nissui Pharmaceutical Co. Other chemicals were purchased from Nakarai Chemicals Ltd., Kyoto.

Flammulina velutipes (enokitake) and *Lentinus edodes* (shiitake) were obtained from a local market.

Methods.

Standard assay conditions for folate-hydrolyzing enzyme .

The FH-enzyme activity was measured under the following conditions. The reaction mixture consists of 0.1 M Tris-HCl buffer, pH 7.1, 1 mM [2-^{14}C]folate (75 nCi) and the enzyme solution in a final volume of 0.1 ml. The mixture was put into a small brown test tube (0.7 x 4.5 cm) with a plug and incubated at 37°C for 1 hr. The reaction was terminated by adding 0.1 ml of ethanol. The mixture was centrifuged at 10,000 x *g* for 10 min. The resulting supernatant (20 µl) was spotted on an Avicel SF cellulose plate (20 x 20 cm). The plate was developed with 0.1 M potassium phosphate buffer, pH 7.0, for 100 min at room temperature. After the plate was dried well in a drying oven at 80°C, a 1-cm region from the origin, on which [2-^{14}C]pteroic acid localized, was scraped off and put into a counting vial containing 10 ml of a scintillation fluid consisting of 0.1 g of POPOP and 4 g of PPO dissolved in one liter of toluene. The radioactivity was measured with a Packard 2425 Tri-Carb Liquid Scintillation Spectrometer. The FH-enzyme activity was shown as the amount of pteroic acid formed per hr under the standard assay conditions.

Another reaction product from folate, *i.e.*, glutamic acid, was determined by a colorimetric method described by Moore.[7] The reaction mixture was diluted to 0.5 ml with water and mixed with 0.5 ml of 2% ninhydrin solution, pH 5.2, containing 0.0625% hydrindantin dissolved in dimethylsulfoxide and 4M lithium acetate (3:1, v/v). The mixture was heated at 100°C for 20 min. The resulting blue-colored solution was diluted with 2.5 ml of 50% ethanol and the ab-

sorbance at 570 nm was measured with a Hitachi 124 Spectrophotometer. The activity of the enzyme which catalyzed the hydrolysis of MTX was also measured by the colorimetric determination of the glutamic acid released.

Preparation of the partially purified FH-enzyme from C. fasciculata. The cells (10g) of *C. fasciculata* were suspended in 50 ml of 0.1 M Tris-HCl buffer, pH 7.1, and disrupted for 5 min on ice with a Kaijo-Denki 20 kHz Ultrasonic Oscillator. The crude extract was centrifuged at 12,000 x *g* for 30 min, and the resulting supernatant was heated at 60°C for 5 min. After the insoluble materials were removed by centrifugation at 12,000 x *g* for 10 min, the resulting supernatant was made to 55% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation at 10,000 x *g* for 10 min and dialyzed overnight against a liter of 0.1 M Tris-HCl buffer, pH 7.1. The specific activity of the final solution was 8-fold higher than that of the crude extract.

Isolation of the reaction products. The crude extract of *C. fasciculata* (29 mg of protein) was incubated with 1 mM folate in 0.1 M Tris-HCl buffer, pH 7.1, at 37°C for 2 hr. The reaction was terminated by adding ethanol. After the mixture was centrifuged at 10,000 x *g* for 10 min, the reaction products, *i.e.*, pteroic acid and glutamic acid, were isolated from the resulting supernatant as follows: Pteroic acid was isolated by a thin-layer chromatography on an Avicel SF cellulose plate and purified by desalting with a Sephadex G-10 column as described in Chapter V.[6] The fractions

showing an absorbance at 280 nm were collected and lyophilized. For isolating glutamic acid, a half of the resulting supernatant described above was concentrated with an evaporator *in vacuo* and was applied to an Avicel SF cellulose column (1.1 x 39 cm) which was previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. Two milliliter fractions were collected. Fractions from #14 to # 17 were pooled and concentrated under vacuum. The residue was suspended in a small amount of 0.2 M sodium citrate-HCl buffer, pH 2.2. After filtrating the solution through a Millipore filter (0.45 μm in pore diameter), glutamic acid was analyzed with a Hitachi KLA-5 automatic amino acid analyzer.

Growth conditions for microorganisms. *Lactobacillus casei* ATCC 7469, *L. arabinosus* ATCC 8014, *L. fermenti* ATCC 9338, *Streptococcus faecalis* R ATCC 8043, *Pediococcus cerevisiae* ATCC 8081 and *Pseudomonas riboflavina* IFO 3140 were grown at 37°C in the medium described by Iwai *et al.*[8] *Bacillus cereus* IFO 3131, *Serratia indica* IFO 3759, *Serratia marcescens* IFO 3048 and *Escherichia coli* B were grown at 30° C by vigorously shaking in the medium described by Iwai *et al.*[9] *Saccharomyces cerevisiae* FKU 1451, *Candida utilis* IFO 0396 and *Hansenula jadinii* IFO 0987 were grown at 30°C in the medium, pH 6.5, containing 1% yeast extract, 2% polypepton and 2% glucose. *Aspergillus niger* M-62, *Penicillum chrysogenum* IFO 4879 and *Neurospora crassa* IFO 6979 were grown at 30°C in the medium, pH 6.0, containing 0.5% glucose, 0.5% polypepton, 0.5% yeast extract and 0.5% sodium chloride. *C. fasciculata* was grown at 25°C in the medium described

by Guttman.[10]

Preparation of the crude extracts. Microbial cells were collected by centrifugation and washed twice with a cold 0.9% saline. The cells (1 g, wet weight) were suspended in 5 ml of 0.1 M Tris-HCl buffer, pH 7.1, and disrupted by sonicating for 5 min. Fungi were disrupted by homogenizing in a glass Potter-Elvehjem homogenizer. Yeast cells (1 g) were disrupted by grinding with 1 g of aluminum oxide in a pestle and mortar. The crude extract was centrifuged at 12,000 x *g* for 30 min. The resulting supernatant was used as the enzyme source.

A male Wister rat (350 g) and a male albino rabbit (2.5 kg) were killed by decapitation. Each 1 g of tissues was homogenized with 10 ml of 0.1 M Tris-HCl buffer, pH 7.1, in a glass Potter-Elvehjem homogenizer. After the homogenate was centrifuged at 12,000 x *g* for 30 min, the resulting supernatant was used for assaying the FH-enzyme activity.

Determination of protein. Protein was determined by the method of Lowry *et al.*[11]

RESULTS AND DISCUSSION

Reaction products of FH-enzyme UV spectra of the reaction product were in good agreement with those of an authentic pteroic acid as shown in Fig. 1. The ninhydrin-positive product was identified as glutamic acid by a thin-layer chromatography as shown in Table I. The product was also confirmed as glutamic acid by its

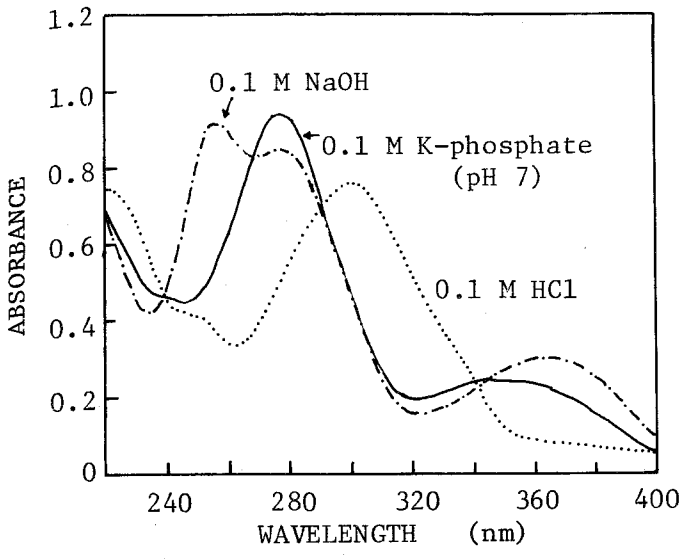


Fig. 1. Ultraviolet Absorption Spectra of the Reaction Product from Folic acid in 0.1 M NaOH, 0.1M HCl and 0.1 M Potassium Phosphate Buffer, pH 7.0.

Table I. *Rf* Values on Thin-Layer Chromatography of the Amino Acid Product.

Folic acid (1 mM) was incubated at 37°C for 2 hr with the crude extract of *C. fasciculata* (29 mg of protein) in 0.1 M Tris-HCl buffer, pH 7.1. The amino acid product was isolated by chromatography on an Avicel cellulose column. The product and authentic L-glutamic acid were spotted on an Avicel SF cellulose plate and developed by various solvent system.

Solvent system*	*Rf* Value	
	Product	L-Glutamic acid
a	0.02	0.05
b	0.40	0.40
c	0.22	0.24
d	0.28	0.28
e	0.31	0.30
f	0.22	0.22
g	0.12	0.11
h	0.95	0.94

* a, ethanol-28% ammonium hydroxide-water, 18:1:1 (by volume); b, methanol-pyridine-water, 20:1:5 ; c, *n*-butanol-pyridine-water, 1:1:1 ; d, *n*-butanol-acetic acid-water, 12:3:5 ; e, phenol solution (160 g in 40 ml of water) ; f, phenol solution-28% ammonium hydroxide, 200:1 ; g, phenol solution-ethanol-28% ammonium hydroxide-water, 150:40:1:10 ; h, 0.1 M potassium phosphate buffer, pH 7.0.

elution time on amino acid analyzer (data not shown).

Stoichiometry of the FH-enzyme reaction

The stoichiometry for FH-enzyme reaction was shown in Table II. The amount of [2-^{14}C] pteroic acid determined by the radioassay was similar to that of glutamic acid determined by the amino acid analyzer, although slightly higher amount of glutamic acid was estimated by the colorimetric assay. These results indicate that FH-enzyme hydrolyzes one mol of folate to each one mol of pteroic acid and glutamic acid. The amount

Table II. Stoichiometry for the Reaction of Folate-hydrolyzing Enzyme.

The reaction mixture (0.3 ml) containing 0.1 M Tris-HCl buffer, pH 7.1, 1 mM [2-^{14}C]folate and the crude extract of *C. fasciculata* was incubated at 37°C for 1 hr. The amounts of protein used in experiment I and II were 450 and 900 μg, respectively. An aliquot (0.1 ml) of the reaction mixture was used for the radioassay of folic acid and pteroic acid, and another aliquot for the colorimetric analysis and the amino acid analysis of glutamic acid.

Experiment	Radioassay		Colorimetric analysis
	Folic acid consumed*(nmol)	Pteroic acid formed* (nmol)	Glutamic acid formed* (nmol)
I	30.5	31.4	36.0
II	51.2	52.3	55.7 (51.6)**

* The amount per 0.1 ml of the reaction mixture.

** Parenthesis indicates the value determined by an amino acid analyzer.

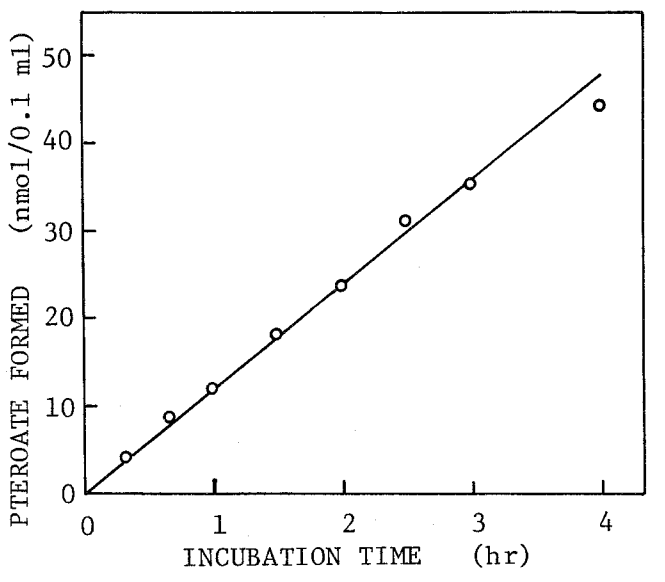


Fig. 2. Formation of Pteroic Acid as a Function of Incubation Time.

The reaction mixture (0.1 ml) containing 1 mM [2-^{14}C]folate (75 nCi), 0.1 M Tris-HCl buffer, pH 7.1, and the partially purified enzyme (30 μg of protein) from *C. fasciculata* was incubated at 37°C for 4 hr. After a defined interval of incubation, [2-^{14}C]pteroic acid formed was separated and measured by the method described in *Methods*.

of the conventional photometric method[1-5] for assaying the FH-enzyme activity.

of [^{14}C]pteroic acid increased linearly for a period of 3 hr-incubation (Fig. 2), and the amount formed per hr was proportional to the concentration of the enzyme (Fig. 3). Above 0.2 nmol of pteroic acid could be detected by this radioassay. The sensitivity of this method was several times higher than that

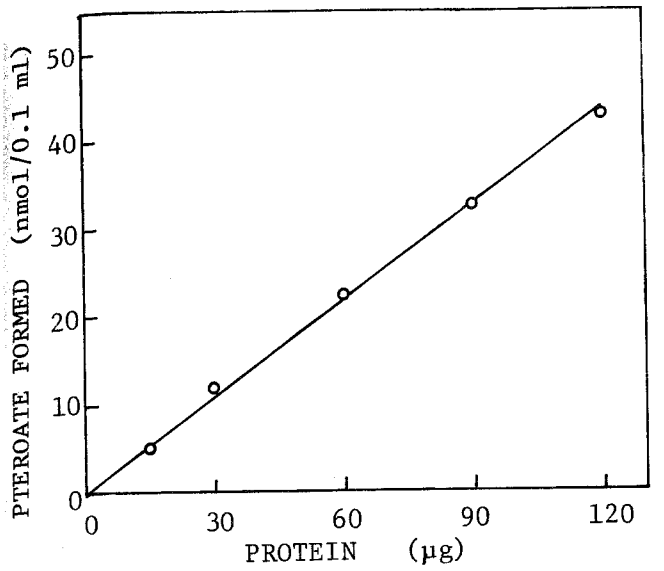


Fig. 3. Formation of Pteroic Acid as a Function of Protein Concentration.
Components of the reaction mixture were the same as those in Fig. 2, except the amount of the enzyme. The mixture was incubated at 37°C for 1 hr. The pteroic acid formed was separated and measured by the method described in *Methods*.

The FH-enzyme activity in microorganisms and mushrooms As shown in Table III, higher activities of FH-enzyme were found in *C. fasciculata* and *N. crassa*. The partially purified FH-enzyme from *C. fasciculata* catalyzed the hydrolysis of MTX to AMPte. This indicates that FH-enzyme in *C. fasciculata* may catalyze both hydrolyzing reactions of folate and MTX as shown by bacterial enzymes.[1-5] *N. crassa* has several intracellular peptidases such as carboxypeptidase and aminopeptidase.[12-14] But, there is no report whether these peptidases catalyze the hydrolysis of folate. *L. casei* had the highest FH-enzyme activity in *Lactobacteriaceae* tested. But, the enzyme activity was slight in *L. arabinosus* ATCC 8014 or *P. cerevisiae* ATCC 8081. *L. casei* metabolizes MTX to AMPte *in vivo*,[15] but *P. cerevisiae* does not.[16] The evidence indicates that FH-enzyme of *L. casei* may take part in the metabolism of MTX as well as that of *C. fasciculata*.[6]

The FH-enzyme activities were slight in *E. coli* B and *A. chroococcum* IFO 12393. Mushrooms had the FH-enzyme activity. The

Table III. Distribution of Folate-Hydrolyzing Enzyme Activity in Microorganisms and Mushrooms

Strain	Specific activity* (nmol/hr/mg of protein)	Total activity* (nmol/hr/g of wet cells or tissue)
Crithidia fasciculata ATCC 12857	72.8	4870
Aspergillus niger M-62	1.7	28
Penicillium chrysogenum IFO 4879	1.4	19
Neurospora crassa IFO 6979	24.2	1020
Serratia indica IFO 3759	1.0	24
Serratia marcescens IFO 3048	0.4	6
Pseudomonas riboflavina IFO 3140	0.8	9
Lactobacillus casei ATCC 7469	1.0	25
Lactobacillus fermenti ATCC 9338	1.1	9
Streptococcus faecalis R ATCC 8043	0.5	3
Bacillus cereus IFO 3131	0.4	13
Lentinus edodes (Shiitake)	1.6	18
Flammulina velutipes (Enokitake)	0.4	4

* The enzyme activity was defined as the amount of pteroic acid formed under the standard assay conditions.

Table IV. Distribution of Folate-Hydrolyzing Enzyme Activity in Mammalian Tissues

Tissue		Specific activity* (nmol/hr/mg of protein)	Total activity* (nmol/hr/g of wet tissue)
Hog	Liver	0.2	27
	Kidney	0.3	21
Rabbit	Brain	0.2	6
	Liver, Kidney, Spleen	trace	<1
Rat	Brain	0.3	9
	Liver	2.7	388
	Kidney	trace	<1
	Spleen	0.2	17

* The enzyme activity was defined as the amount of pteroic acid formed under the standard assay conditions.

FH-enzyme activity were not detected in yeasts such as *S. cerevisiae* FKU 1451, *C. utilis* IFO 0396 and *H. jadinii* IFO 0987. A proteolytic enzyme from *S. cerevisiae*, *i.e.*, carboxypeptidase Y,[17] could

not hydrolyze folate and MTX at any pH range between 4 and 9. The FH-enzyme activity was not detected in *Euglena gracilis*.

The FH-enzyme in mammalian tissues As shown in Table IV, the highest FH-enzyme activity was found in the crude homogenate of rat liver. The homogenate also had the hydrolyzing activity of MTX to AMPte (data not shown). AMPte, excreted in rat urine and feces as a metabolite of MTX, is supposed to be formed by bacterial enzymes in rat intestine.[18,19] However, the present result suggests the possibility that rat liver may produce AMPte from MTX. The FH-enzyme activity was also found in hog liver, but it was slight in rabbit liver.

By using the sensitive radioassay, the auther has found that the FH-enzyme activity is widely distributed in biological cells and tissues.

REFERENCES

1) C. C. Levy and P. Goldman, *J. Biol. Chem.*, 242, 2933 (1967).
2) P. Goldman and C. C. Levy, *Proc. Natl. Acad. Sci. U.S.A.*, 58, 1299 (1967).
3) J. L. McCullough, B. A. Chabner and J. R. Bertino, *J. Biol. Chem.*, 246, 7207 (1971).
4) A. G. Pratt, E. J. Crawford and M. Friedkin, *J. Biol. Chem.*, 243, 6367 (1968).
5) A. M. Albrecht, E. Boldizsar and D. J. Huchison, *J. Bacteriol.*, 134, 506 (1978).

6) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 1847(1983).

7) S. Moore, *J. Biol. Chem.*, 243, 6281 (1968).

8) K. Iwai, O. Okinaka and H. Yokomizo, *Vitamins*, 35, 387 (1967).

9) K. Iwai, M. Kobashi and H. Fujisawa, *J. Bacteriol.*, 104, 197(1970).

10) H. N. Guttman, "Pteridine Chemistry", ed. by W. Pfleiderer and E. C. Taylor, Pergamon Press, London, 1964, p. 255.

11) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193, 265 (1951).

12) D. Siepen, P. H. Yu and M. R. Kula, *Eur. J. Biochem.*, 56, 271(1975).

13) D. Siepen and M. R. Kula, *FEBS Letters*, 66, 31 (1976).

14) S. Tan and G. A. Marzluf, *J. Bacteriol.*, 137, 1324 (1979).

15) Y. S. Shin, K. U. Buehring and E. L. R. Stokstad, *J. Biol. Chem.*, 249, 5772 (1974).

16) K. U. Bühring and E. Fölsch, *Biochim. Biophys. Acta*, 421, 22(1976).

17) R. Hayashi, S. Moore and W. H. Stein, *J. Biol. Chem.*, 248, 2296 (1973).

18) D. S. Zaharko and V. T. Oliverio, *Biochem. Pharmacol.*, 19, 2923 (1970).

19) D. M. Valerino, *Res. Commun. Chem. Pathol. Pharmacol.*, 4, 529 (1972).

f) H. Oe, M. Kohashi and K. Iwai, *J. Nutr. Sci. and Vitaminol.*, 29, 523 (1983).

Chapter VII

Purification and Some Properties of Folate-hydrolyzing Enzyme from *Crithidia fasciculata*

As described in Chapter VI, the author has developed a sensitive method for analyzing the folate-hydrolyzing enzyme (FH-enzyme) which catalyzed the hydrolysis of folic acid to pteroic acid and glutamic acid.[1] The author has also found the enzyme activity in the cell-free extracts of *C. fasciculata* ATCC 12857 and other biological cells and tissues.[1,2] The similar enzyme activity has been found inductively in soil bacteria such as *Alcaligenes faecalis*,[3] *Pseudomonas* sp.[4,5] and *Flavobacterium* sp.[6,7] which can grow on folate compounds as a sole source of carbon and nitrogen. But, *Crithidia* had constitutively the FH-enzyme activity. In the present chapter, the auther purified the FH-enzyme from *C. fasciculata* and compared the properties of the enzyme with those of the inductive bacterial enzymes.

MATERIALS AND METHODS

Materials. The following chemicals were obtained from the specified manufacturers: [2-^{14}C]Folic acid potassium salts (58.2 m Ci/mmol) from the Radiochemical Centre (Amersham); bovine pancreas α-chymotrypsinogen *A*, pepsin, ovalbumin, bovine serum albumin, aminopterin and DFP from Sigma Chemical Co.; methotrexate from Lederle Ltd.; bovine serum γ-globulins from Mann Research Lab.;

human serum γ-globulin from Nutritional Biochemical Co.; 5-methyl, 5-formyl, and 10-formyltetrahydrofolates from Eisai Chemicals Co.; 8-hydroxyquinoline, β-mercaptopropionic acid and α,α'-dipyridyl from Wako Pure Chemical Industries Ltd.; Sephadex G-200 from Pharmacia Fine Chemicals; Avicel SF cellulose plates (20 x 20 cm) from Funakoshi Pharmaceutical Co. Bathophenanthroline disulfonic acid disodium salt, bathocuproine disulfonic acid disodium salt, *o*-phenanthroline, α,α',α''-tripyridyl, pABG and other chemicals were obtained from Nakarai Chemical Ltd., Kyoto.

Growth conditions for C. fasciculata. *C. fasciculata* ATCC 12857 was statically cultivated at 25°C in 200 ml-Erlenmeyer flask containing 50 ml of the culture medium described by Guttman.[8] The cell growth was measured by an increase of turbidity at 675 nm using a Coleman Model 6-20 Spectrophotometer. The cells were harvested at a defined interval of growth by centrifugation at 3,000 x *g* for 10 min and washed twice with a cold 0.9% (w/v) saline solution. The cells were suspended in 2 to 5 ml of 0.1 M Tris-HCl buffer, pH 7.1, and disrupted for 2 min on ice with a Kaijo-Denki 20 kHz Ultrasonic Oscillator. The suspension was centrifuged at 12,000 x *g* for 30 min. The resulting supernatant solution was used for assaying the FH-enzyme activity.

For a large-scale preparation of cells, *C. fasciculata* was cultivated in the culture medium (120 liters) at 25°C for 60 hr by stirring at 300 rpm and by aerating at a flow rate of one liter/min. The cells were harvested by continuous centrifugation at 10,000 x

g with a High Speed Centrifuge CM-60RN, Tomy Seiki Co. Ltd., and washed with the saline solution. The cells were stored at -20°C until use.

Standard assay conditions for the FH-enzyme activity. The FH-enzyme activity was measured by radioassay described in Chapter VI.[1] The reaction mixture consists of 0.1 M Tris-HCl buffer, pH 7.1, 2 mM [2-^{14}C]folate (50 μCi) and the enzyme solution in a final volume of 0.1 ml. The mixture was incubated at 37°C for 1 hr. When non-radioactive compounds and pABG were used as the substrate, the FH-enzyme activity was measured by a colorimetric method using a ninhydrin reagent described in Chapter VI.[1] The enzyme activity was defined as amounts of pteroate formed per hr or as changes in absorbance at 570 nm per hr under the standard assay conditions.

Purification of the FH-enzyme.

1) Preparation of crude extract. All procedures were carried out at 0 - 4°C. The *Crithidia* cells of 500 g (wet weight) were suspended in 2.5 liters of 0.1 M Tris-HCl buffer, pH 7.1, containing 0.1 mM $CoCl_2$ and homogenized with glass beads (0.25 - 0.5 mm in diameter) using a Dyno-Mill KDL, Shinmura Enterprise Co. The homogenate was centrifuged at 12,000 x *g* for 20 min.

2) Heat treatment. The resulting supernatant solution (2.3 liters) was heated at 60°C for 5 min and centrifuged at 12,000 x *g* for 20 min. The resulting supernatant solution (2.25 liters) was diluted twice with water. The recovery of the enzyme activity was almost 100%.

3) DEAE-cellulose column chromatography. The diluted solution was put on a DEAE-cellulose column (5.7 x 32 cm) which had been previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.1, containing 0.1 mM $CoCl_2$ (hereafter this buffer will be referred to as the Buffer). The enzyme was eluted by a linear Tris gradient with 1.8 liters of the Buffer in the mixing chamber and 1.8 liters of 0.6 M Tris-HCl buffer, pH 7.1, containing 0.1 mM $CoCl_2$, in the reservoir. Fractions of 17 ml were collected. Tubes from #100 to #130 were pooled and the solution was dialyzed against 10 liters of the Buffer. The dialyzed solution (560 ml) was put on a DEAE-cellulose column (2.5 x 26 cm) which had been equilibrated with the Buffer. The column was eluted by a linear Tris gradient with 600 ml of the Buffer in the mixing chamber and 600 ml of 0.5 M Tris-HCl buffer, pH 7.1, containing 0.1 mM $CoCl_2$ in the reservoir. Fractions of 7 ml were collected. Tubes from #70 to #90 were pooled, and the solution was concentrated with a collodion bag (Ultrahülsen UH 100) and dialyzed against 0.1 M Tris-HCl buffer, pH 7.1, containing 0.1 mM $CoCl_2$. The recovery of the enzyme activity was about 14%.

4) Sephadex G-200 column chromatography. The dialyzed solution (ca. 2 ml) was subjected to a chromatography on Sephadex G-200 column (1.5 x 90 cm) which had been equilibrated with 0.1 M Tris-HCl buffer, pH 7.1, containing 0.1 mM $CoCl_2$. Fractions of 1.8 ml were collected. Tubes from #28 to #38 were pooled and the solution was concentrated to a small volume with a collodion bag. The recovery of the enzyme activity were 95%.

5) Preparative polyacrylamide gel electrophoresis. A aliquot (0.2 ml) of the concentrated solution was put on a 7.5% polyacrylamide gel column (2 x 5 cm) which was prepared by the method of Williams and Reisfeld.[9] A preparative gel electrophoresis was performed at a current of 8 mA at 4°C for 10 hr with a Fuji-Riken Furicoverer II. Overrunning protein fractions were continuously washed out from the gel into the running elution buffer of 10 mM Tris-HCl, pH 7.1, at a flow rate of 0.4 ml/min. Fractions of 1.86 ml were collected. Tubes from #27 to #30 were pooled and the solution was concentrated with a collodion bag. The recovery of the enzyme activity was 16%.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide gel electrophoresis was carried out at pH 8.9 in a 7.5% gel for 90 min with a current of 2 mA per tube by the method of Davis.[10] Polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS was carried out in a 10% gel for 5 hr with a current of 8 mA per tube by the method of Weber and Osborn.[11] Protein was stained with Coomassie brilliant blue G-250.

Estimation of the molecular weight. The molecular weight of the enzyme was estimated by a gel filtration on a Sephadex G-200 column (1.5 x 90 cm) which had been equilibrated with 0.1 M Tris-HCl buffer, pH 7.1, containing 0.1 mM $CoCl_2$. Tryptophanase from *Proteus rettgeri* (220,000 daltons), bovine γ-globulin (160,000 daltons), human γ-globulin (153,000 daltons), bovine serum albumin (63,000 daltons) and ovalbumin (43,000 daltons) were used as molecu-

lar markers.

Determination of protein. Protein was determined by the method of Lowry *et al.* [12] using bovine serum albumin as a standard and by an absorbance at 280 nm using a Hitachi 124 Spectrophotometer.

RESULTS

Time course of the FH-enzyme formation The formation of the FH-enzyme activity during growth is shown in Fig. 1. The maximum formation was observed at 80 hr after cultivation. After that time, total enzyme activity was extremely decreased. The cells cultivated until their concentration reached T=60 - 50 % were used for purifying the enzyme.

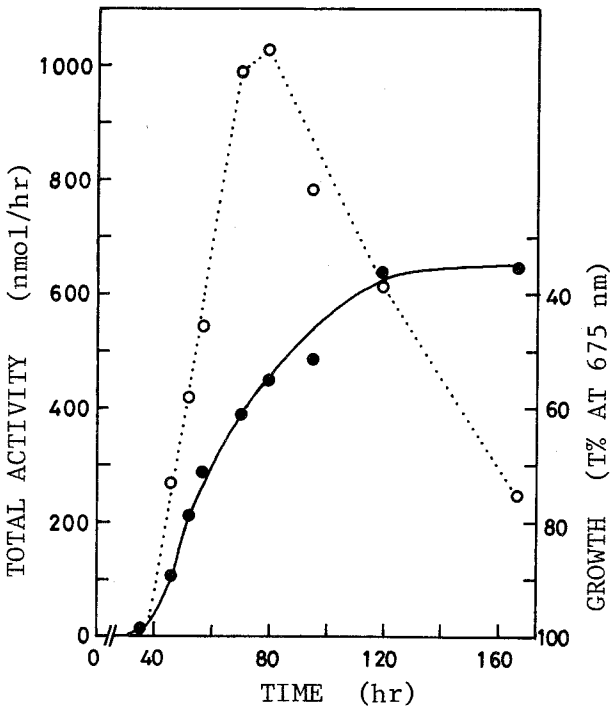


Fig. 1. Time Course of the Folate-hydrolyzing Enzyme Formation and Growth of *C. fasciculata.*
C. fasciculata was statically cultivated at 25°C in 200 ml-Erlenmeyer flask containing 50 ml of the culture medium. The enzyme activity in the cells obtained at a defined interval of growth was assayed under standard assay conditions described in *Methods*. The activity was shown as amounts of pteroate formed per cells at 37°C for 1 hr (o---o). The growth was shown as transmittance (T%) at 675 nm (●—●).

Purification of FH-enzyme The elution profile of the FH-enzyme activity on polyacrylamide gel electrophoresis is shown in Fig. 2. Table I summarizes the purification procedure for the FH-

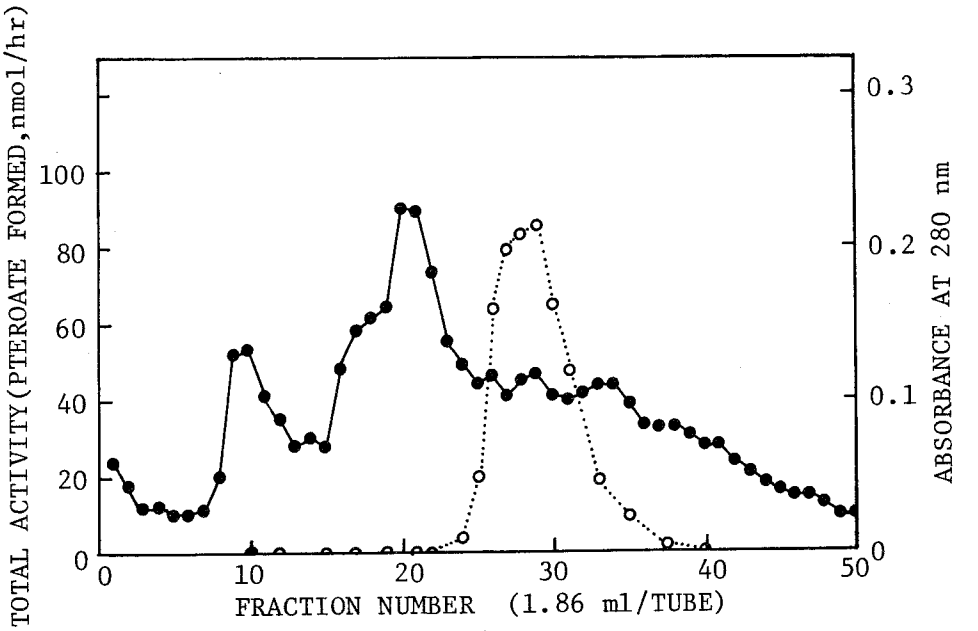


Fig. 2. Preparative Polyacrylamide Gel Electrophoresis of the Folate-hydrolyzing Enzyme.

Enzyme fraction (0.2 ml) obtained from the chromatography on Sephadex G-200 column was put on a 7.5% polyacrylamide gel column. The protein was eluted as described in *Methods*. (●——●), absorbance at 280 nm; (o----o), total activity per tube.

Table I. Summary of Purification of the Folate-hydrolyzing Enzyme from *C. fasciculata*.

Purification step	Protein (mg)	Activity* (nmol/hr)	Specific activity* (nmol/hr/mg protein)	Yield (%)
Crude extract	47,190	1,920,700	40.7	100.0
Heat treatment	7,750	1,919,900	247.7	100.0
1st DEAE-cellulose	2,966	870,070	293.3	45.3
2nd DEAE-cellulose	709	273,500	385.8	14.2
Sephadex G-200	270	260,540	965.0	13.6
Electrophoresis	21.3	42,281	1985	2.2

* The enzyme activity was estimated as amounts of pteroate formed under standard assay conditions.

enzyme. The enzyme was purified 49-fold from the crude extract with a yield of 2.2%. The final preparation was electrophoretically homogeneous (Fig. 3). The specific activity was 1985 nmol of pteroate formed per mg of protein per hr at 37°C.

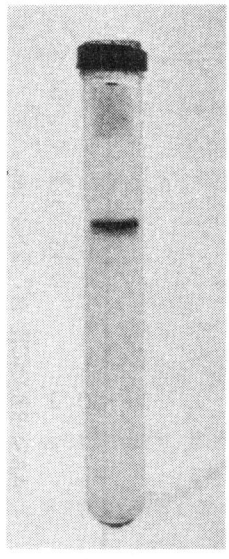


Fig. 3. Polyacrylamide Gel Electrophoresis of the Purified Folate-hydrolyzing Enzyme. Electrophoresis was performed using a 7.5% gel as described in *Methods*. The protein was stained with Coomassie brilliant blue G-250.

Molecular weight and subunit structure The molecular weight of the enzyme was estimated to be 200,000 daltons by gel filtration on a Sephadex G-200 column. The SDS-treated enzyme showed a single band which molecular weight was 51,000 daltons. These results indicate that the enzyme consists of 4 subunits with the same molecular size.

Effect of pH on the enzyme activity and stability

The pH-activity profile of the enzyme is shown in Fig. 4A. The optimum pH was 7.0. At pH 6.8, the enzyme activity showed the lowest in potassium phosphate buffer. The enzyme was stable below pH

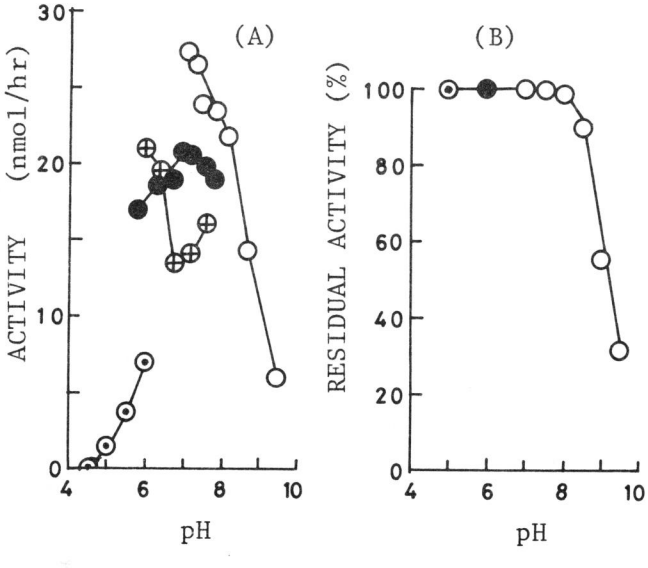


Fig. 4. Effect of pH on the Folate-hydrolyzing Enzyme Activity (A) and pH Stability of the Enzyme (B).
(A): The enzyme (14 μg of protein) was incubated with each 0.1 M buffer under standard assay conditions. The buffers used were: ⊙ , sodium citrate; ● , Tris-maleate; ⊕ , potassium phosphate; ○ , Tris-HCl.
(B): The enzyme was preincubated with the buffer described at (A) at 37°C for 2 hr, and the residual activity was measured under standard assay conditions.

8, but unstable over pH 8.5 (Fig. 4B).

Effect of temperature on the enzyme activity and stability

As shown in Fig. 5A, the optimum temperature for the reaction was 50°C. The enzyme lost rapidly its activity by heat treatment over 60°C. The enzyme was stable at pH 7.0 below 50°C as shown in Fig. 5B. Unlike the crude enzyme, the purified enzyme lost 9 and 19% of the activity by heat treatment at 60°C for 5 and 10 min, respectively.

Substrate specificity As shown in Table II, the purified FH-enzyme hydrolyzed various folate compounds. MTX and aminopterin were more effectively used as substrate than folate. But, the reduced forms of folate compounds such as dihydrofolate and 10-

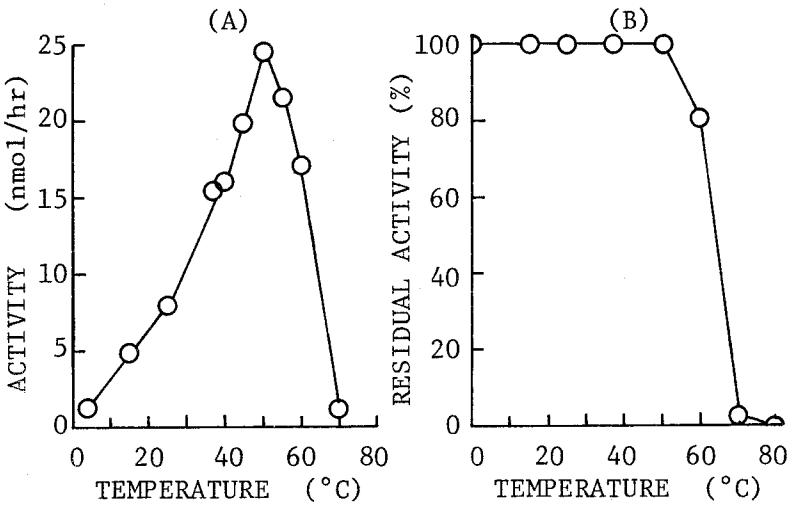


Fig. 5. Effect of Temperature on the Folate-hydrolyzing Enzyme Activity (A) and on Stability of the Enzyme (B).

(A): The enzyme (8 μg of protein) was incubated at the temperature indicated, and the enzyme activity was measured under standard assay conditions.

(B): After the enzyme was preincubated at a defined temperature for 10 min, the remaining activity was measured.

Table II. Substrate Specificity of Folate-hydrolyzing Enzyme from *C. fasciculata*.

The enzyme (8.2 μg of protein) was incubated with each substrate (2 mM) under standard assay conditions. The enzyme activity was assayed by the colorimetric method using a ninhydrin reagent described in Chapter VI.[1)]

Substrate	Enzyme activity* ($\Delta A_{570\ nm}$/hr)	Relative activity
Folate	0.103	1.00
Aminopterin	0.309	3.00
Methotrexate	0.287	2.79
Dihydrofolate	0.047	0.46
10-Formyltetrahydrofolate	0.057	0.55
5-Formyltetrahydrofolate	0.035	0.34
5-Methyltetrahydrofolate	0.023	0.22
Pteroyl-γ,γ-diglutamyl-glutamic acid (teropterin)	0	-
p-Aminobenzoylglutamic acid	0.123	1.19

* The enzyme activity was defined as a change in absorbance at 570 nm per hr.

formyl, 5-formyl, and 5-methyltetrahydrofolates were less effectively used than folate. pABG was used actively as folate. The enzyme did not have the conjugase activity which catalyzed the hydrolysis of the γ-linkage of pteroyl-γ,γ-diglutamylglutamate (teropterin). *Km* values for folate, MTX, aminopterin and pABG were calculated to be 0.13, 0.46, 0.40 and 0.43 mM, respectively, from double-reciprocal plots of reaction rate *versus* substrate concentration.

Reaction products The reaction products formed from folate were identified as pteroate and L-glutamate by the same methods as described in Chapter VI.[1)] The enzyme hydrolyzed stoichiometrically folate to pteroate and L-glutamate. Thus, one mol of the enzyme hydrolyzed 397 mol of folate to equal mols of pteroate and L-glutamate under standard assay conditions. The reaction products from MTX or aminopterin were also identified as 4-amino-4-deoxy-10-methylpteroate or 4-amino-4-deoxypteroate and L-glutamate, respectively, from the similar results of their behaviors on thin-

layer chromatography and their ultraviolet and infrared absorption spectra as reported by Levy and Goldman[4] (data not shown).

Effect of metal ions and salts As shown in Table III, Hg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+} inhibited strongly the enzyme activity in this order. Cr^{3+} and Yb^{3+} also inhibited. But, the following salts did not affect the activity: $MgCl_2$, $CaCl_2$, $SnCl_2$, $BaCl_2$, $MnCl_2$, $FeCl_2$, $FeCl_3$ and $AlCl_3$ at the concentration of 0.1 mM, and LiCl, NaCl and KCl at the concentration of 0.1 M. Inorganic and organic sodium salts such as pyrophosphate, borate, orthophosphate and citrate at the concentration of 20 mM also inhibited the activity (Table III).

Table III. Effect of Various Inorganic and Organic Salts on the Folate-hydrolyzing Enzyme Activity.

The enzyme (8 μg of protein) was preincubated at 37°C for 10 min with each salt in 0.1 M Tris-HCl buffer, pH 7.1, and incubated with 2 mM [2-^{14}C]folate (50 μCi) under standard assay conditions.

Addition	Concentration (mM)	Relative activity (%)
None	-	100.0
$NiCl_2$	0.1	92.8
$CuCl_2$	0.1	10.4
$ZnCl_2$	0.1	40.2
$SrCl_2$	0.1	92.7
$PbCl_2$	0.1	30.2
$CdCl_2$	0.1	24.0
$HgCl_2$	0.1	0.4
$CrCl_3$	0.1	70.1
$YbCl_3$	0.1	88.7
$NaNO_3$	20	88.3
Na_2SO_4	20	89.3
Na_2CO_3	20	95.4
$Na_2B_4O_7$	20	67.5
Na_3PO_4	20	73.5
$Na_4P_2O_7$	20	10.1
Na-Acetate	20	88.3
Na_2-Maleate	20	89.2
Na_2-Malate	20	86.4
Na_2-Tartarate	20	95.4
Na_2-Glutamate	20	90.1
Na_2-Aspartate	20	88.8
Na_3-Citrate	20	78.9

Effect of chemical reagents

The enzyme activity was inhibited by chelating reagents such as α,α',α''-tripyridyl, bathophenanthroline disulfonate, bathocuproine

Table IV. Effect of Chelating Reagents on the Folate-hydrolyzing Enzyme Activity.

The enzyme (8 μg of protein) was preincubated at 37°C for 10 min with 0.1 mM of each reagent in 0.1 M Tris-HCl buffer, pH 7.1, and incubated under standard assay conditions.

Chelating reagent	Relative activity (%)
None	100.0
α,α',α''-Tripyridyl	19.0
o-Phenanthroline	52.7
Bathophenanthroline disulfonate	45.8
Bathocuproine disulfonate	50.3

disulfonate and *o*-phenanthroline (Table IV). EDTA, rubeanic acid, α, α'-dipyridyl, 8-hydroxyquinoline and β-mercaptopropionic acid at the concentration of 0.1 mM inhibited 15, 11, 7, 2 and 1% of the enzyme activity, respectively. When the enzyme was preincubated with 0.8 mM bathophenanthroline disulfonate at 37°C for 30 min and dialyzed against 5 mM Tris-HCl buffer, pH 7.0, for 6 hr, the enzyme lost 81% of its activity. However, the enzyme activity was recovered to 44% by the addition of 0.1 mM $CoCl_2$. The addition of other metal ions did not recover the enzyme activity.

pCMB at the concentration of 0.02 mM inhibited 50% of the enzyme activity. 2-Mercaptoethanol at 10 mM also inhibited 45% of the activity. However, 0.2 mM PMSF, 1 mM DFP or 15.2 μM pepstatin A did not affect the enzyme activity.

DISCUSSION

The present study was undertaken to characterize the FH-enzyme which was purified homogeneously from the crude extract of *C. fasciculata* ATCC 12857. The enzyme hydrolyzes various folate compounds at a neutral pH (Table II and Fig. 4A) as do soil bacterial peptidases such as carboxypeptidase (CPase) $G^{4)}$ and $G_1{}^{5)}$ of *Pseudo-*

monad, and folate amidase[6] and CPase[7] of *Flavobacteria*. The enzyme activity of *C. fasciculata* depends on the structure of the pteridine moiety of folate compounds. The reduced forms were hydrolyzed only 0.2 to 0.5-fold of folate, and diaminopteridine derivatives such as aminopterin and MTX were more effectively hydrolyzed than folate. But, the *Km* values for folate and MTX of the *Crithidia* enzyme showed 2 to 130 times higher than those of the three bacterial CPases.[4,5,7] Like the *Pseudomonas* CPase G_1[5] and the *Flavobacterium* CPase,[7] the *Crithidia* enzyme also hydrolyzed pABG, but did not hydrolyze the γ-glutamyl linkage of pteroyl-γ,γ-diglutamylglutamate. This indicates that the *Crithidia* enzyme may have a specificity for spliting the α-glutamyl linkage of folate compounds and pABG.

The two *Pseudomonas* CPases require Zn^{2+} for their activities.[4, 13] The *Crithidia* enzyme activity was inhibited by various chelating reagents (Table IV), heavy metal ions, and organic and inorganic anions (Table III). But, the activity of the bathophenanthroline-treatment enzyme was partially recovered by adding $CoCl_2$. These evidences suggest that the *Crithidia* enzyme may be a kind of metalloprotein and require Co^{2+} for its activity.

Like the two *Flavobacterium* folate amidase[6] and CPase,[7] the *Crithidia* enzyme was inhibited by 2-ME. Although folate amidase is only sensitive to phosphate buffer and bacterial CPase is also to citrate buffer, the *Crithidia* enzyme activity was affected by inorganic and organic salts including pyrophosphate, borate, phosphate and citrate. There is no information about inhibition of bacterial

enzymes by pCMB and peptidase inhibitors such as DFP, PMSF and pepstatin A. The *Crithidia* enzyme was sensitive to pCMB.

Activities of proteinases and aminopeptidases have been found in crude homogenates of protozoa, *Trypanosomatidae*, such as *Crithidia*, [14-17] *Trypanosoma*[14,17-21] and *Leishmania*.[14] However, there are no reports whether these proteases of protozoa may hydrolyze folate compounds.

REFERENCES

1) H. Oe, M. Kohashi and K. Iwai, *J. Nutr. Sci. and Vitaminol.*, 29, 523 (1983).

2) H. Oe, M. Kohashi and K. Iwai, *Agric. Biol. Chem.*, 47, 1847(1983).

3) W. S. McNutt, *Arch. Biochem. Biophys.*, 101, 1 (1963).

4) C. C. Levy and P. Goldman, *J. Biol. Chem.*, 242, 2933 (1967).

5) J. L. McCullough, B. A. Chabner and J. R. Bertino, *J. Biol. Chem.*, 246, 7207 (1971).

6) A. G. Pratt, E. J. Crawford and M. Friedkin, *J. Biol. Chem.*, 243, 6367 (1968).

7) A. M. Albrecht, E. Boldizsar and D. J. Hutchison, *J. Bacteriol.*, 134, 506 (1978).

8) H. N. Guttman,"Pteridine Chemistry," ed. by W. Pfleiderer and E. C. Taylor, Pergamon Press, London, 1964, p. 255.

9) D. E. Williams and R. A. Reisfeld, *Annal. N. Y. Acad. Sci.*, 121, 373 (1964).

10) B. J. Davis, *Annal. N. Y. Acad. Sci.*, 121, 404 (1964).

11) K. Weber and M. Osborn, *J. Biol. Chem.*, 244, 4406 (1969).

12) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193, 265 (1951).

13) B. A. Chabner and J. R. Bertino, *Biochim. Biophys. Acta*, 276, 234 (1972).

14) R. F. Steiger, F. V. Hoof, J. Bontemps, M. Nyssens-Jadin and J. -E. Druetz, *Acta Tropica*, 36, 335 (1979).

15) J. McLaughlin, H. S. Injeyan and E. Meerovitch, *Can. J. Biochem.*, 54, 365 (1976).

16) F. B. ST. C. Palmer, *Comp. Biochem. Physiol.*, 47B, 515 (1974).

17) N. N. Sukhareva-Nemakova, M. V. Chuenkova, V. G. Kreir and N. S. Egorov, *Biol. Nauki*, 4, 99 (1981).

18) R. F. Steiger, F. R. Opperdoes and J. Bontemps, *Eur. J. Biochem.*, 105, 163 (1980).

19) F. R. Opperdoes and R. F. Steiger, *Mol. Biochem. Parasitol.*, 4, 311 (1981).

20) M. Torruella, B. M. Franke de Cazzulo, J. C. Engel, A. M. Ruiz, E. L. Segura and J. J. Cazzulo, *Comp. Biochem. Physiol.*, 70B, 463 (1981).

21) P. Rautenberg, R. Schadler, E. Reinwald and H.-J. Risse, *Mol. Cell. Biochem.*, 47, 151 (1982).

Chapter VIII

Properties of Folate-hydrolyzing Enzyme of *Crithidia fasciculata* as a Carboxypeptidase

In Chapter VII, the auther described the purification and some properties of the FH-enzyme from *C. fasciculata* ATCC 12857.[1] The enzyme catalyzed the hydrolysis of the amide linkage of folate compounds, as did CPase G[2,3] and CPase G_1[4,5] of *Pseudomonad*, and CPase[6] and folate amidase[7] of *Flavobacteria*. But some physical properties of the *Crithidia* enzyme differed from those of bacterial enzymes.[1] The author found preliminarily that the *Crithidia* enzyme hydrolyzed also Z-Gly and Z-Ala. This suggests that the *Crithidia* enzyme may show a lack of specificity for the α-carboxy terminal amino acids. In the present chapter, the author describes the substrate specificity of the FH-enzyme using some synthetic Z-dipeptides and Z-amino acids.

MATERIALS AND METHODS

Materials. Z-Tyr, Z-Phe-Tyr, Z-Tyr-Glu, Z-Gly-Phe-NH_2, Boc-D-Phe, Gly-Phe, Leu-enkephalin and angiotensin II (human) were obtained from Protein Research Foundation, Osaka. CPase A (bovine pancreas) was from Sigma Chemical Co. Z-D-Phe and Z-D-Glu were synthesized from carbobenzoxy chloride and D-phe, and from carbobenzoxy chloride and D-Glu, respectively, in alkaline solution by the method described by Izumiya.[8]

Standard assay conditions for FH-enzyme. The FH-enzyme activity was assayed by a colorimetric method using a ninhydrin reagent described in Chapters VI[9] and VII,[1] and defined as changes in absorbance at 570 nm per hr.

RESULTS AND DISCUSSION

Effect of Z-dipeptides, Z-amino acids and peptides on the FH-enzyme Table I shows that the FH-enzyme hydrolyzes various Z-L-dipeptides and Z-L-amino acids, as well as folate and MTX.[1] Z-Gly-Tyr, Z-Phe-Tyr and Z-Phe-Ala were hydrolyzed 30 times more effectively than folate. Carboxy-terminal glutamic acids such as Z-Gly-Glu, Z-Phe-Glu, Z-Glu-Glu and Z-Tyr-Glu were 4 to 14 times more effectively used as the substrate than folate. All of Z-L-amino acids tested in the present study were hydrolyzed. Z-Phe and Z-Tyr were 3 and 4 times more effective substrates than folate, respectively. The enzyme (3 μg of protein) released 1.88 mmol of leucine and 0.05 mmol of phenylalanine after incubating with 2 mmol of Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) at 37°C for 11 hr. However, the following peptides were ineffective as the substrate: Z-Gly-Gly, Z-Gly-Leu, Z-Ala-Gly, Z-Phe-Pro, Z-Gly-Phe-NH_2, Bz-Gly-Arg, Gly-Asp, Gly-Glu, Gly-Tyr, Asn-Glu, Boc-D-Phe, Z-D-Phe, Z-D-Glu, angiotensin II and carboxymethylated ribonuclease. The FH-enzyme activity in the presence of each substrate was maximum at pH 7.0 as shown in the presence of folate.[1] The *Km* values for Z-Phe-Ala, Z-Gly-Tyr, Z-Phe-Glu, Z-Glu-Glu, Z-Phe and Z-Tyr were 0.39, 1.43, 0.65, 0.11,

Table I. Substrate Specificity of Folate-hydrolyzing Enzyme from *Crithidia fasciculata*

The FH-enzyme (1.1 μg of protein) was incubated with each substrate (1 mM) in 0.1 M Tris-HCl buffer, pH 7.1, at 37°C for 1 hr. The enzyme activity was measured by a colorimetric method using a ninhydrin reagent.[9]

Substrate*	Enzyme activity ($\Delta A_{570\ nm}$/hr)	Relative activity
Folate	0.014	1.0
Z-Phe-Gly	0.120	8.6
Z-Phe-Ala	0.443	31.6
Z-Phe-Glu	0.140	10.0
Z-Phe-Phe	0.380	27.1
Z-Phe-Tyr	0.448	32.0
Z-Tyr-Gly	0.045	3.2
Z-Tyr-Glu	0.057	4.1
Z-Glu-Glu	0.073	5.2
Z-Glu-Phe	0.013	0.9
Z-Glu-Tyr	0.021	1.5
Z-Ala-Phe	0.158	11.3
Z-Gly-Glu	0.189	13.5
Z-Gly-Phe	0.302	21.6
Z-Gly-Tyr	0.487	34.8
Z-Gly	0.007	0.5
Z-Ala	0.018	1.3
Z-Asp	0.017	1.2
Z-Glu	0.018	1.3
Z-Glu-γ-OEt	0.020	1.4
Z-Phe	0.047	3.4
Z-Tyr	0.059	4.2

* L-Amino acids were used.

** The enzyme activity was defined as a change in absorbance at 570 nm.

0.25 and 0.32 mM, respectively.

The two *Pseudomonas* enzymes hydrolyze some α-carboxy terminal glutamate peptides including Z-Glu, Glu-Glu, Gly-Glu and Tyr-Glu, as well as folate and MTX, but fail to hydrolyze another carboxy terminal amino acid peptides.[3,4] The present data showed that *Crithidia* enzyme hydrolyzed various α-carboxy terminal amino acid peptides and especially, Z-dipeptides containing aromatic amino

acid. This property is much different from those of the soil bacterial enzymes.[2-7] It is well-known that the pancreatic CPase A hydrolyzes considerably more slowly Z-amino acids than the corresponding Z-Gly-amino acids.[10] The substrate specificity of the FH-enzyme is similar to that of the bovine CPase A. However, this CPase A did not hydrolyze folate and MTX at any pH range between 4 and 9 (data not shown). These results indicate that the FH-enzyme of *C. fasciculata* is a new type of intracellular CPase, having the folate hydrolase activity.

REFERENCES

1) H. Oe, M. Kohashi and K. Iwai, Submitted in *Agric. Biol. Chem.*.

2) C. C. Levy and P. Goldman, *J. Biol. Chem.*, 242, 2933 (1967).

3) P. Goldman and C. C. Levy, *Proc. Natl. Acad. Sci. USA.*, 58, 1299 (1967).

4) J. L. McCullough, B. A. Chabner and J. R. Bertino, *J. Biol. Chem.*, 246, 7207 (1971).

5) B. A. Chabner and J. R. Bertino, *Biochim. Biophys. Acta*, 276, 234 (1972).

6) A. M. Albrecht, E. Boldizsar and D. J. Hutchison, *J. Bacteriol.*, 134, 506 (1978).

7) A. G. Pratt, E. J. Crawford and M. Friedkin, *J. Biol. Chem.*, 243, 6367 (1968).

8) N. Izumiya, *Tanpakushitsu Kakusan Koso*, 9, 306 (1964).

9) H. Oe, M. Kohashi and K. Iwai, *J. Nutr. Sci. and Vitaminol.*, 29, 523 (1983).

SUMMARY

Chapter I;

MTX and aminopterin promoted the growth-rate of *C. fasciculata* ATCC 12857 as well as biopterin and folate. The growth curve obtaind by the 0.1 μM MTX-medium coincided with that by the 0.85 nM biopterin-medium. The DFR activity in the cells grown in the MTX-medium was the same as that in the biopterin-medium.

Chapter II;

Three different DFRs (I, IIa and IIb) were separated from the cell-free extract of *C. fasciculata* ATCC 12857. The major DFR IIa was purified 2744-fold by column chromatographies on DEAE-Sephadex, CM-Sephadex, Sephadex G-150 and folate-Sepharose 4B. The final preparation was homogeneous in electrophoretic analysis. Its molecular weight was estimated by gel filtration to be 110,000 daltons and consisted of two subunits with the same molecular weight of 58,000 daltons. The optimum pH was 7.0. The enzyme activity depended on dihydrofolate and NADPH. Other folate derivatives, unconjugated pteridines and NADH were ineffective. *Km* values for dihydrofolate and NADPH were 1.1 and 2.7 μM, respectively. One mol of the enzyme reduced 755 mol of dihydrofolate per min at 30°C. The enzyme activity was inhibited by pCMB, NEM and urea. It was also inhibited by naturally occurring folates such as 10-formyltetrahydrofolate, 5-formyltetrahydrofolate and folate, anti-folates

such as MTX, aminopterin, pyrimethamine and trimethoprim, and by NADP. One mol of the enzyme was stoichiometrically inhibited by 0.81 - 0.86 mol of MTX and by 0.88 mol of aminopterin.

Chapter III;

PtR:DFR (fraction IIb) obtained from *C. fasciculata* was purified 60-fold. The molecular weight was estimated to be 110,000 daltons by Sephadex G-150 gel filtration. The enzyme reduced the neopterin isomers (L-*threo*-, L-*erythro*-, D-*threo*- and D-*erythro*-), 6-hydroxymethylpterin, 6-methylpterin and xanthopterin as well as dihydrofolate and dihydropteroate. The reaction with L-*threo*-neopterin had a double pH optimum (6.0 and 4.5), while that with 6-hydroxymethylpterin occurred over a pH range between 6.5 and 4.5. The optimum pH's using dihydrofolate and dihydropteroate as the substrates were 6.8 and 7.0, respectively. *Km* values for L-*threo*-neopterin, 6-hydroxymethylpterin, dihydrofolate and dihydropteroate were 3.5, 3.4, 4.8 and 0.9 μM, respectively. The reaction was dependent on NADPH, requiring two mol of NADPH for reduction of one mol of L-*threo*-neopterin. *Km* values for the NADPH in assays with L-*threo*-neopterin, 6-hydroxymethylpterin, dihydrofolate and dihydropteroate were 11, 5.9, 5.9 and 2.1 μM, respectively. The reaction product was the tetrahydro form of each pteridine compound. The enzyme activity was inhibited by biopterin, folate, MTX, pyrimethamine, trimethoprim and NADP, as well as by pCMB, NEM and urea. These evidences suggest that this enzyme is a new type of DFR.

Thus, the name, pteridine reductase:dihydrofolate reductase (PtR:DFR), is suggested for this enzyme.

Chapter IV;

The activity of PtR:DFR relative to the structures of 4 stereoisomers (L-*threo*-, L-*erythro*-, D-*threo*-, and D-*erythro*-) of neopterin is described. The data show that the activity of the enzyme depended heavily on the configuration of OH at C-1' of the trihydroxypropyl side chain of neopterin as a substrate.

Chapter V;

The uptake and metabolism of MTX were examined in *C. fasciculate* ATCC 12857. When the cells grown in a medium containing biopterin were incubated with MTX, the uptake of MTX by the cells was completely within 1 min, and the amounts of MTX per 10^8 cells were maintained between 19 and 37 fmol/min throughout the remaining growth period. In contrast, the uptake of folate was linear for at least 20 min, and the uptake rate of folate by the cells varied from 31 to 247 fmol/min/10^8 cells during the growth. The folate uptake was inhibited by MTX and aminopterin. When *C. fasciculata* was cultivated in a medium containing MTX, the MTX taken up by the cells was metabolized to several compounds. One of the metabolites was isolated from the cells and the culture supernatant, using DEAE-cellulose and Sephadex G-10 column chromatographies, and identified as 4-amino-4-deoxy-10-methylpteroic acid (AMPte) on the basis

of its behavior on thin-layer chromatography, and of its ultraviolet and infrared absorption spectra. An enzyme activity converting MTX to AMPte was found in the cell-free extract of *C. fasciculata*.

Chapter VI;

A sensitive radioassay method has been developed to quantitate the activity of the folate-hydrolyzing enzyme (FH-enzyme) which catalyzes the hydrolysis of folic acid to pteroic acid and glutamic acid. The method is based on analyzing [2-^{14}C]pteroic acid separated by a thin-layer chromatography on an Avicel SF cellulose plate using 0.1 M potassium phosphate buffer, pH 7.0, as a solvent. This method was found to be more sensitive than a conventional photometric method to determine tha activity of the FH-enzyme. High activities of the enzyme were found in *Crithidia fasciculata* ATCC 12857, *Neurospora crassa* IFO 6979 and rat liver. Smaller activities of the enzyme were widely distributed in other microbial cells and mammalian tissues.

Chapter VII;

The folate-hydrolyzing enzyme (FH-enzyme) was purified 49-fold from the crude extract of *C. fasciculata* ATCC 12857 by heat treatment, column chromatographies on DEAE-cellulose and Sephadex G-200, and preparative polyacrylamide gel electrophoresis. The final preparation was electrophoretically homogeneous. The enzyme had a molecular weight of 200,000 daltons and consisted of 4 identical

subunits which molecular weight was about 51,000 daltons. The enzyme hydrolyzed more effectively aminopterin, MTX and pABG than folate. The enzyme hydrolyzed more weakly the reduced folates, dihydrofolate and 10-formyltetrahydrofolate than folate. The enzyme did not act on pteroyl-γ,γ-diglutamylglutamate. The optimum pH for the reactions with each substrate described above was 7.0. *Km* values for folate, MTX, aminopterin and pABG were 0.13, 0.46, 0.40 and 0.43 mM, respectively. The enzyme activity was inhibited by 2-ME, pCMB, chelating reagents such as α,α',α''-tripyridyl and bathophenanthroline, divalent cations such as Hg^{2+}, Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+}, and by pyrophosphate and orthophosphate.

Chapter VIII;

The FH-enzyme of *C. fasciculata* hydrolyzed various synthetic Z-dipeptides such as Z-Phe-Ala and Z-Gly-Tyr, and Z-L-amino acids such as Z-Phe, Z-Tyr and Z-Glu, as well as folate and MTX. The data shows that the enzyme is a lack of substrate specificity for the α-carboxy terminal amino acids and is a new type of carboxypeptidase.

From the results described in Chapter I through VIII, it is concluded that *C. fasciculata* ATCC 12857 metabolizes MTX and aminopterin as well as folate to compounds which may be used effectively to promote its growth. Figure 1 summarizes the proposal of MTX and folate metabolism in this protozoan. Brooker[1)] has reported

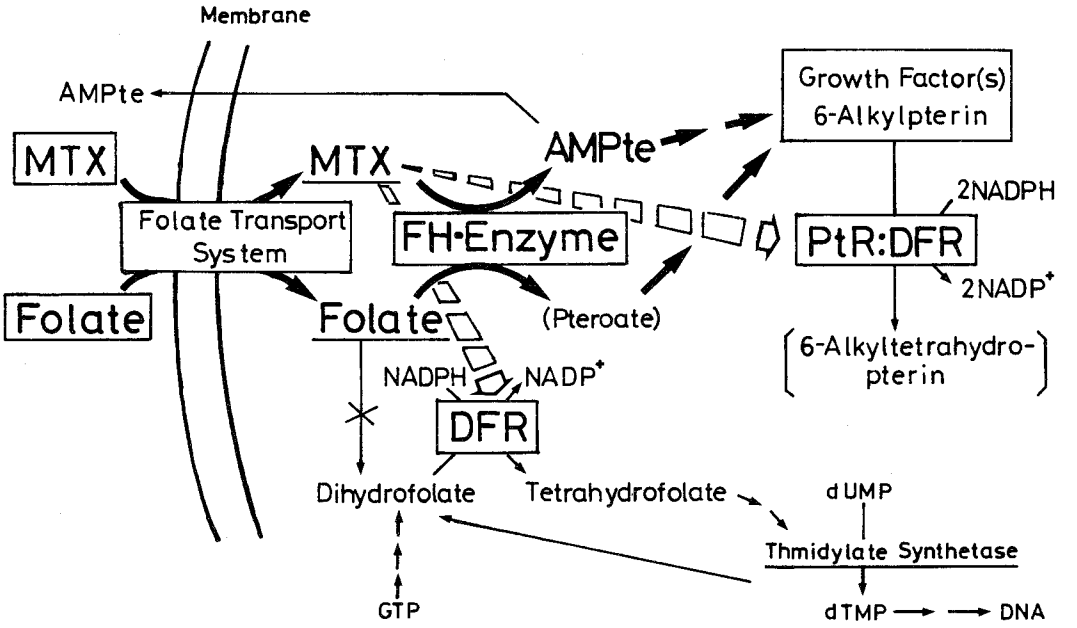


Fig. 1. A Schema of MTX and Folate Metabolism of *C. fasciculata*.

that many microtubules are located under trilaminar cell membrane of *C. fasciculata*. The microtubules are known to be involved in the receptors and carrier systems. *C. fasciculata* has two different carrier systems for transporting biopterin and folate,[2] and the biopterin carrier system is thought to be located in these pellicular microtubules,[3] while it is still unknown about the folate system. As described in Chapter V and reported by Rembold and Vaubel,[2] the folate transport in this protozoan was an energy-dependent active process, and the uptake of MTX and aminopterin inhibited significantly the folate transport. These results indicate that MTX and aminopterin uptake may share the carrier site for folate located in the microtubules.

MTX inhibited stoichiometrically the DFR activity and compet-

ively the PtR:DFR activity of *C. fasciculata*, *in vitro*, as did a number of DFRs from various sources[4] (see Chapters II and III). On the contrary, MTX inhibited only slightly the DFR activity, *in vivo*, and promoted the growth of this protozoan as well as 6-alkylpterins, such as biopterin,[5-8] neopterin[8,9] and 6-hydroxymethylpterin[8] (see Chapter I). The growth promoting effect of MTX was observed at the same concentrations as did folate.[5,6] In Chapter V, *C. fasciculata* metabolized MTX to at least four compounds including AMPte *in vivo* and excreted partially AMPte to the culture medium. In Chapters V through VIII, the author also found from *C. fasciculata* a new intracellular carboxypeptidase which catalyzed effectively the hydrolysis of the amide linkage of folate and MTX and was tentatively named as the FH-enzyme. Rembold and Eder[10] have reported that aminobiopterin (2,4-diamino-6-[L-*erythro*-1,2-dihydroxypropyl]pteridine) has a growth activity of *C. fasciculata* similar to that of biopterin. These evidences suggest strongly that *C. fasciculata* may metabolize MTX to 6-alkylpterin compounds *via* the hydrolyzing reaction to AMPte and the deaminating reaction of the amino group at C-4 of the pteridine ring.

Folate is thought to be metabolized with the same process as MTX in this protozoan, based on the following results: 1) Unlike mammalian and bacterial sources,[4] any evidence of folate reduction was unable to be detected in the crude or partially purified fractions of *C. fasciculata*, including four reductases (see Chapter II). 2) As bacterial[11] and mammalian cells,[12] *C. fasciculata* had a GTP

cyclohydrolase activity which catalyzed the initial reaction of the *de novo* biosynthetic pathway of dihydrofolate. 3) Folate is metabolized *in vivo* to 6-hydroxymethylpterin and biopterin which have a greater promoting effect on the growth of *C. fasciculata*.[13] From the reasons described above, it is concluded that the FH-enzyme catalyzes the initial reaction of the folate and MTX metabolic pathway and also play a role in the detoxication of MTX in *C. fasciculata*.

The new type of reductase, tentatively named as PtR:DFR, catalyzed the reduction of various unconjugated pteridine compounds as well as dihydrofolate and dihydropteroate (see Chapter III). As in the mammalian nervous system, serotonin has been detected in *C. fasciculata*.[14] Serotonin is synthesized *de novo* from tryptophan by way of a tryptophan hydroxylase reaction requiring tetrahydropteridines as a cofactor.[15,16] Serotonin and 5-hydroxytryptophan are active in sparing the unconjugated pteridine requirement of *C. fasciculata*.[17] The author found in the crude extract of this protozoan a tyrosine hydroxylase activity requiring the tetrahydropteridine compound. From these evidences, PtR:DFR may play a role of the reduction of 6-alkylpteridine compounds for requiring the aromatic amino acid hydroxylations in *C. fasciculata*.

REFERENCES

1) B. E. Brooker, *Parasitol.*, 72, 259 (1976).

2) H. Rembold and A. Vaubel, *Hoppe-Seyler's Z. Physiol. Chem.*, 351,

1277 (1970).

3) H. Rembold and T. Langenbach, *J. Protozool.*, 25, 404 (1978).

4) R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines," North-Holland Pub. Co. Amsterdam, London, 1969, p.139.

5) G. W. Kidder and B. N. Dutta, *J. Gen. Microbiol.*, 18, 621 (1958).

6) H. A. Nathan and H. B. Funk, *Am. J. Clin. Nutr.*, 7, 375 (1959).

7) H. Rembold and H. Metzger, *Hoppe-Seyler's Z. Physiol. Chem.*, 329, 291 (1962).

8) V. C. Dewey and G. W. Kidder, *Methods in Enzymol.*, 18B,618(1971).

9) E. L. Patterson, R. Milstrey and E. L. R. Stokstad, *J. Am. Chem. Soc.*, 80, 2018 (1958).

10) H. Rembold and J. Eder, *Tetrahedron*, 23, 1387 (1967).

11) J. J. Yim and G. M. Brown, *J. Biol. Chem.*, 251, 5087 (1976).

12) E. M. Gal, J. M. Nelson and A. D. Sherman, *Neurochem. Res.*, 3, 69 (1978).

13) G. W. Kidder, V. C. Dewey and H. Rembold, *Arch. Mikrobiol.*, 59, 180 (1967).

14) K. Janakidevi, V. C. Dewey and G. W. Kidder, *Arch. Biochem. Biophys.*, 113, 758 (1966).

15) G. W. Kidder, "Chemical Zoology," ed. by M. Florkin and B. Scheer, Vol. I, Academic Press, New York, 1966.

16) A. Ichiyama, S. Nakamura, Y. Nishizuka and O. Hayaishi, *J. Biol. Chem.*, 245, 1699 (1970).

17) T. Kato, T. Yamaguchi, T. Nagatsu, T. Sugimoto and S. Matsuura, *Biochim. Biophys. Acta*, 611, 241 (1980).

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